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414 Rec'd PCT/PTO 06 OCT 2000

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PTO-1390 (REV. 1/98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER	U.S. APPLICATION NO. (If known, see 37 CFR 1.5
102.174	09/673166

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR99/00792	April 6, 1999	April 7, 1998

TITLE OF INVENTION LIPOPEPTIDES INDUCING T LYMPHOCYTIC CYTOTOXICITY BEARING AT LEAST ONE AUXILIARY T EPITOPE AND USES FOR VACCINATION

APPLICANT(S) FOR DO/EO/US

LE GAL et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [ ] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [XX] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). Unexecuted
10. [ ] A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

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Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Modified claims; Modified Drawings (12 sheets); French International Preliminary Examination Report; PCT/IB/304; PCT/IB/308

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U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/673166	PCT/FR99/00792	102.174

17. [X] The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1070.00	$1000.00
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $930.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $790.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $720.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $98.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$1000.00
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	21 - 20 =	1	x $18.00	$
Independent claims	- 3 =		x $82.00	$ 18.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$

TOTAL OF ABOVE CALCULATIONS =	$1018.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28). +	$
SUBTOTAL =	$1018.00
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$
TOTAL NATIONAL FEE =	$1018.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +	$
TOTAL FEES ENCLOSED =	$1018.00
Amount to be refunded:	$
charged:	$

a. [X] PTO Form 2038 is enclosed. ~~A check in the amount of $ _____ to cover the above fees is enclosed.~~

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2275 A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Bierman, Muserlian and Lucas
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20311
PATENT TRADEMARK OFFICE

Charles A. Muserlian
SIGNATURE

Charles A. Muserlian
NAME

19,683
REGISTRATION NUMBER

09/673166
529 Rec'd PCT/PTO 06 OCT 2000

Our Ref.: 102.174

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
PCT/FR99/00792	:	PCT Date: April 6, 1999
LE GAL et al	:	
Serial No.:	:	
Filed: Concurrently Herewith	:	
For: LIPOPEPTIDES...FOR VACCINATION	:	

600 Third Avenue
New York, NY 10016

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the application as follows:

IN THE SPECIFICATION:

Page 1, before line 1, insert

--This application is a 371 of PCT/FR99/00792 filed April 6, 1999.--

IN THE MODIFIED CLAIMS:

Claim 3, line 1, change "one of claims 1 to 2" to --claim 3--.

Claim 4, line 1, change "one of claims 1 to 3" to --claim 1--.

Claim 5, line 1, change "one of claims 1 to 3" to --claim 1--.

Claim 6, line 1, change "any one of claims 1 to 3" to --claim 1--.

Claim 7, line 1, change "one of claims 1 to 6" to --claim 1--.

Claim 8, line 1, change "one of claims 1 to 6" to --claim 1--.

Claim 9, line 1, change "one of claims 1 to 8" to --claim 1--.

Claim 10, line 1, change "one of claims 1 to 9" to --claim 1--

Claim 11, line 1, change "one of claims 1 to 10" to --claim 1--.

Claim 12, line 1, change "one of claims 1 to 10" to --claim 1--.

Claim 13, line 1, change "one of claims 1 to 12" to --claim 1--.

Claim 14, line 1, change "one of claims 1 to 13" to --claim 1--.

Claim 15, line 1, change "one of claims 1 to 14" to --claim 1--.

Claim 16, line 1, change "one of claims 1 to 14" to --claim 1--.

Claim 17, line 1, change "one of claims 1 to 16" to --claim 1--.

Claim 18, line 1, change "one of claims 1 to 17" to --claim 1--.

Claim 19, line 1, change "one of claims 1 to 17" to --claim 1--.

Claim 20, line 3, change "one of claims 1 to 17" to --claim 1--.

Claim 21, line 2, change "one of claims 1 to 17" to --claim 1--.

REMARKS

The amendment is presented to insert reference to the PCT

application, remove multiple dependency from the claims.

Respectfully submitted,
BIERMAN, MUSERLIAN AND LUCAS

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CAM:sd
Enclosure: Return Receipt Postcard

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Lipopeptides inducing T lymphocytic cytotoxicity bearing at least one auxiliary T epitope and uses for vaccination

This invention relates to lipopeptides inducing T lymphocytic cytotoxicity and bearing at least one auxiliary T epitope. Moreover, it relates to the use of said lipopeptides as vaccine.

There are two types of immune response : the humoral response due to antibodies and the cytotoxic response due to $CD8^+$ T lymphocytes.

An efficient cytotoxic response requires the antigens to be presented to the cytotoxic $CD8^+$ T lymphocytes (CTL), in association with molecules of class I of the Major Histocompatibility Complex (MHC), but also to the auxiliary $CD4^+$ T lymphocytes (HTL) in association with molecules of class II of the MHC.

The use of lipopeptides for inducing a cytotoxic response, i.e. the in vivo generation of cytotoxic T lymphocytes has already been disclosed. More particularly, lipopeptides made of a peptide portion comprising from 10 to 40 amino-acids and of a lipid portion that can be derived from fatty acids or steroid groups are disclosed in FR-90 15 870 (publication 2,670,787) (Institut Pasteur de Lille, Institut Pasteur, INSERM).

Such lipopeptides are liable to induce a cytotoxic response. It was however suitable to make them able to also induce an auxiliary T response, the importance thereof being well known for inducing and maintaining the cytotoxic response.

Upon Applicants' knowledge, only one publication under the name of **VITIELLO and al.** (1995, J. Clin. Invest., 95, 341-349) has mentioned the possibility of combining on a same lipopeptide molecule a CTL epitope and an epitope inducing an auxiliary response (T-HELPER or HTL epitope).

The synthesis of two lipopeptides is disclosed in such publication. The former is made of the portion 18-27 of the hepatitis B virus core, as CTL epitope, of the portion 830-843 of the tetanic toxin, as auxiliary T epitope, and of two palmitoyl chains. The authors note the induction of a lymphocytic T cytotoxicity.

The second lipopeptide is made of the NP 147-155 epitope of the murine influenza virus, of an auxiliary T epitope and of lipid chains.

However, the lipopeptides disclosed in such publication show a low solubility, resulting from the presence on the one hand of the lipid portion and on the other hand of hydrophobic peptide patterns. It should be noted on that respect that the products disclosed by these authors are stored in mother solutions in concentrated DMSO and diluted extemporaneously for injection in a buffered aqueous solution.

Such a high hydrophobicity makes it difficult to produce solutions of such lipopeptides and thereby considerably restrict the sterilization possibilities through filtering, which is the method commonly used due to its easy processability. It moreover considerably restricts any characterization possibility.

The problem to be solved was therefore to reduce the hydrophobicity of the multi-epitope lipopeptides while maintaining the accessibility to the implementation needed to convey the CTL epitope pattern to the class I CMH.

The present invention aims at solving this problem.

The Inventors have now found that it was possible not only to increase the affinity for water of the lipopeptide molecules, but also to restrict the interactions between the different epitopes, through introducing hydrophilic sequences of aminoacids, so-called spacers, within such molecules.

The object of the present invention is thus lipopetides comprising at least one auxiliary T epitope, at least one CTL epitope and at least one lipid moiety characterized in that the epitopes and the lipid portion on the one hand and the epitopes on the other hand are independently separated by amino-acid sequences, so-called spacers, comprising linkages of generally charged amino-acids in a neutral medium, providing the lipopeptide hydrophilic.

Preferably, the spacers are accessible to a proteolytic implementation through the proteasome, prior to the release of the CTL epitope pattern. Such accessibility may be enhanced as disclosed by **OSSENDORP and al.** (1996, Immunity, 5, 115-124).

The spacers preferably comprise from 1 to 10, more preferably from 2 to 4 amino-acids, the amino-acid quantity being enough to obtain generally charged spacers in a neutral medium. Preferably, at least one of

the spacers comprises arginines and/or glycines. Arginines have the advantage of being charged at physiologic pHs and of creating sites being particularly sensible to the proteolytic cleavage upon epitopes being implemented.

Glycines have the advantage to allow the introduction, within the peptide portion of the lipopeptide, of an optional site for a converging synthesis by moiety coupling.

According to a particularly advantageous embodiment of the invention, at least one of the spacers has one of the following sequences :

GLY ARG or

ARG GLY ARG.

The glutamic acid and/or the aspartic acid also show advantageous properties applicable to the production of spacers according to the present invention (access to the natural proteolytic degradation and introduction of ionized carboxylate functions in a physiological medium at neutral pH).

These amino-acids, entering the spacer sequence, may be advantageously replaced by their derivatives or by other functionally equivalent amino-acids.

These amino-acids, within the spacers, are liable to be separated by not hindered amino-acids, i.e. having short side chains, that can be alanine besides glycine. The presence of such short chain amino-acids makes easy proteolysis.

A cysteine, or an alkyl chain functionalized by a thiol group, able to create an optional site of simple chemical ligation between two peptide moieties due to formation of non peptide covalent bonds may also be introduced into the spacer. It is thereby possible to create a disulfide bond between two different peptides, each comprising a thiol function or a thioether bond, in which case one of these two peptides must be functionalized by an alkyl halogenide.

Both peptides may also be linked by forming a thiazolidine heterocycle, one of the peptides bringing an aldehyde function. This aldehyde function can be easily and selectively generated in the form of an alpha-oxo-acyl group, obtained through a periodic oxidation of a serine, a threonine or a cysteine introduced into a N-terminal position in a peptide moiety.

It is also possible to combine both peptides by using the aldehyde reactivity with weak bases or ligation methods through forming an oxime (by reaction between an aldehyde function and an amino-oxyacetyl function), a hydrazone (by reaction between an aldehyde function and a hydrazide or an arylhydrazine). Such basic reactions are mentioned in the publication by James Tam and Jane Spetzler (Biomed. Pep., Prot. and Nucleic Acids (1995), 1, 123-132).

A ligation method consisting in generating a hydrazone bond from an alkylhydrazine (generated by N-amination of an amine precursor) and from a partner functionalized by an alpha-oxo-acyl group has recently been developed.

Hydrazinopeptides are synthesized by N-amination according to Klinguer et al. (Tet. Lett. (1996), 37, 7259-7262). This methodology enables to convert any amine function of the peptide into a hydrazine function. The hydrazine function may thereby be positioned in a N-terminal position or on the side chain of an amino-acid positioned in any point in the sequence. The converted amine function may be an alpha, epsilon amine function of a lysine or aminocaproic acid, a delta amino function of an ornithine or any primary or secondary amine function.

The aldehyde peptides are generated such as disclosed by Tam and Spetzler (Biomed. Pep., Prot. and Nucleic Acids (1995), 1, 123-132). The serine, threonine or cysteine moiety precursors of the alpha-oxo acyl group may lie in N-terminal position or on any amine function of a side chain as long as such moieties are not present in N-terminal position in the epitopes being considered.

The lipid group may be carried indifferently by either the hydrazinopeptide or the aldehyde peptide.

For a better understanding of the present invention, an auxiliary T epitope means an amino-acid sequence able to combine with at least a HLA receptor of class II.

By CTL epitope, it is meant an amino-acid sequence able to combine with at least a HLA receptor of class I.

The auxiliary T epitopes able to combine with several different HLA receptors of class II are called multivalent auxiliary epitopes (multivalent HTL).

The lipopeptides according to the present invention preferably comprise a linkage of :

- a lipid portion;
- a first spacer;
- an auxiliary T epitope;
- a second spacer; and
- a CTL epitope.

They may also comprise :

- a lipid portion;
- a first spacer;
- an auxiliary T epitope;
- a second spacer;
- a CTL epitope;
- a third spacer; and
- a second CTL epitope.

The various auxiliary epitopes and CTL may be present in different arrangements in the amino-acid linkage. Thus, an auxiliary epitope may lie between two CTL epitopes, from which it will be separated by two spacers.

The lipid portion of the lipopeptide is advantageously obtained by adding a lipid pattern on an alpha amine function of a peptide or on a reactive function of the side chain of an amino-acid of the peptide portion, such as an epsilon amine or thiol function. It may comprise one or several optionally branched or insaturated chains derived from C_{10}-C_{20} fatty acids or a steroid derivative.

Advantageously, the lipid portion comprises at least two chains derived from C_{10}-C_{20} fatty acids or fatty alcohols, also mutually linked through one or more amino-acids. The lipid portion may therefore comprise two palmitic acid chains, linked to the NH_2, alpha and epsilon groups of a lysine.

The lipid portion may also be made of, or comprise a moiety of palmitic, oleic, linoleic, linolenic, 2-aminohexadecanoic acids, pimelautide or trimexautide.

The non lipid portion comprises from 15 to 100, preferably from 15 to 50 amino-acids. The amino-acid number depends upon the epitope number forming the non lipid portion of the lipopeptide, and their sizes.

The auxiliary T epitope is advantageously an epitope adapted to combine with several different HLA receptors of class II, i.e. a multivalent epitope. It is preferably the auxiliary epitope made of the 830-843 peptide of the tetanic toxin having the following sequence :

QYIKANSKFIGITE

The glutamine (Q) of this sequence may optionally be acetylated.

Other multivalent HTL epitopes may be the multivalent epitope of the haemagglutinin (**PREVOST-BLONDEL et al.**, 1995, J. Virol., vol. 62, n° 12, pages 8046-8055) or also the PADRE epitope (**ALEXANDER et al.**, 1994, Immunity, 1, 751).

As far as the CTL is concerned, it may be any epitope adapted to activate the cytotoxic $CD8^+$ T lymphocytes.

It is preferably a CTL epitope of a protein presented by a tumor cell, in particular by a melanoma, of a HIV protein, a hepatitis B virus (VBH) or the papillomavirus, or the protein p53 as well.

It may be more particularly one of the following epitopes :

- epitopes of the BCR-ABL protein, resulting from the BCR-Abelson translocation (chronic myeloid leukemia) such as mentioned in table 1,
- epitopes of the p53 protein, such as those mentioned in table 2; the epitopes of the p53 protein may additionally be taken out of sequences 25-35, 63-73, 129-156, 149-156, 187-205, 187-234, 226-264 or 249-264 of this protein,
- epitopes of the E_6 or E_7 proteins of the human papillomavirus (VPH), such as those mentioned in table 3,
- epitopes of the proteins of the HIV-1 virus such as those mentioned in table 4,
- epitopes of the melanoma or other tumors, such as those mentioned in tables 5, 6 and 7 and, more particularly, epitopes of the melan-A/mart-1 antigen of the melanoma.

Other plurivalent CTL epitopes showing an association capacity with HLA of class I may be those included in the 43-57 peptide of HPV (GQAEPDRAHNIVTF) containing HLA A2, A24, B7 and B18 epitopes.

The CTL epitopes may additionally be those of parasitic antigens and, more particularly, those of a protein of the intrahepacytory stadium of *Plasmodium falciparum*.

The bond between the lipid portion and the non lipid portion is preferably performed through the ε-NH_2 group of the first amino-acid of the non lipid portion. It may be however performed with any other means, and more particularly with the α-NH_2 of a lysine.

The lipopeptides according to the present invention may be administered to the patients to be treated, in particular to persons to be vaccinated, diluted in an appropriate solvent, such as for instance a physiologically acceptable buffer. They may however put into a galenic form, compatible with an administration through parenteral, sublingual, intrapulmonary or transdermal route.

They may administered with any administration mode allowing an efficient action. This mode will be selected depending upon the disease to be treated. In a non limitative way, the lipopeptides may be more particularly administered through injection or sublingual route.

Consequently, another object of the present invention is the use of such lipopeptides for producing a medicine or a vaccine, either preventive or curative, for inducing a specific immune response and, more particularly, for inducing an immune response against cancers such as melanoma, HIV and VBH viruses, papillomaviruses, p53 or malaria.

A further object of the present invention is a pharmaceutical composition characterized in that it comprises a pharmacologically active quantity of one or more of the above-described lipopeptides, as well as pharmaceutically compatible excipients.

It should finally be noted that the CTL epitopes described in the present specification are themselves objects of the present invention.

The present invention will be illustrated although with no limitation by the following examples.

Fig. 1 shows the recognition of monopalmitoyl and dipalmitoyl lipopeptides according to the invention, comprising the 27-35 Mart epitope, by a lineage of specific cytotoxic T lymphocytes of said epitope in a Elispot-interferon gamma (IFN-γ) test.

Figs. 2A and 2B illustrate the recognition of the lipopeptides mentioned in Fig. 1, by two clones of infiltrating lymphocytes of melanomas, LT8 and LT12 respectively, specific of the 27-35 Mart epitope.

Fig. 3 shows the induction of an auxiliary T activity by the TT 830-843 peptide constitutive of the lipopeptides according to the invention.

- **Fig. 3a** : secretion of γ-interferon by lymphatic gland cells of HLA-A2 transgenic mice being injected with :

(A) NaCl buffer,

(B) Mp-MART lipopeptide,

(C) Dp-MART lipopeptide.

- **Fig. 3b** : proliferation of lymphatic gland cells of HLA-A2 transgenic mice in response to the same antigens.

Fig. 4 illustrates the induction of the γ-interferon secretion by lymphatic gland cells of HLA-A2 transgenic mice in response to :

(a) a mixture of 27-35 MART and TT 830-843 peptides,

(b) Mp-MART lipopeptide,

(c) Dp-MART lipopeptide.

Fig. 5 illustrates the lipopeptide formula comprising a hydrazone bond.

Fig. 6 shows the antigenicity of the HIV epitopes and the corresponding lipopeptides thereof.

HIV seropositive patients' PBMC's have been incubated with the selected peptides (plain bars) or the corresponding lipopeptides (dotted bars). The activation of the specific CTLs has been followed by using the ELISPOT test specific for γ-interferon. The codes allocated to each patient and their HLA type are indicated. The data are representative for two independent experiments.

Fig. 7 shows the immunogenicity of the epitopes limited to HLA-A2 and HLA-A3 and the corresponding lipopeptides.

(a) The indicated peptides or lipopeptides have been injected to HLA-A2 transgenic mice. Before injection at the tail base, the peptides have been mixed with TT 830-843 auxiliary T epitope and emulsified in Freund's incomplete medium; the lipopeptides are simultaneously diluted in a physiological buffer. The production of specific CTL has been determined with the ELISPOT test specific for the γ-interferon, after incubating lymphatic gland cells with target cells of Jurkat-A2 K^B type being pre-incubated in the presence (dashed bar) or in the absence (plain bar) of the indicated CTL epitope.

(b) The indicated peptides or lipopeptides have been injected at the tail base of HLA-A3 transgenic mice. The production of specific CTLs has been determined by the ELISPOT test specific for the γ-interferon, after incubating lymphatic gland cells with EBV target cells expressing the pre-incubated HLA-A3 in the presence (dashed bar) or in the absence (plain bar) of the NEF 73-81 epitope of HIV.

The results shown are representative of three independent experiments.

Fig. 8 shows the need for an auxiliary T epitope of the TT 830-843 type for inducing CTL.

(a) Monopalmitoyl lipopeptides comprising CTL POL 476-484 epitope of HIV free (POL 1 lipo) or not (TT-POL1 lipo) from TT 830-843 auxiliary T epitope have been diluted in a physiological buffer and injected at the tail base of HLA-A2 transgenic mice. The results are representative for two independent experiments.

(b) Lymphatic gland cells of mice immunized with the TT-POL1 monopalmitoyl lipopeptide or the TT 830-843 monopalmitoyl lipopeptide emulsified in Freund's incomplete medium or injected with Freund's incomplete medium alone, have been co-cultivated for 72 hours in the presence (dashed bar) or in the absence (plain bar) of 30 µg/ml of the TT 830-843 auxiliary T epitope.

The cell proliferation has been followed by measuring the ^3H thymidine addition. The results are expressed as the radioactivity mean value (cpm) of the cultures (3 wells per point) for at least three experiments.

Example 1 : Synthesis of dipalmitoylated lipopeptides with and without a spacer and physico-chemical properties

1. Lipopeptide syntheses

The series of the three following lipopeptides has been synthetized.

Lipopeptide no. 1

Pam-K(Pam)-SS-QYIKANSKFIGITE-AAA-AAGIGILTV

Lipopeptide no. 2

Pam-K(Pam)-SS-QYIKANSKFIGITE-RGR-AAGIGILTV

Lipopeptide no. 3

Pam-K(Pam)-GR-QYIKANSKFIGITE-RGR-AAGIGILTV

Such lipopeptides comprise a lipid end made of two palmitoyl (Pam) moieties linked to the NH_2 group of a lysine, the auxiliary epitope of the tetanic toxin (830-843 peptide) and a CTL epitope recognized in mart-1 by the HLA A2.1 receptor. The 18-27 Hbc epitope disclosed in the above mentioned publication by **VITIELLO et al.** is also recognized by HLA A2.1 and shows a similar hydrophobicity to the selected pattern in the above mentioned lipopeptide series.

The difference lies in the amino-acid sequences included on the one hand between the lipid portion and the auxiliary epitope and on the other hand between the auxiliary epitope and the CTL epitope. Such series has been produced in order to assess the possibility to obtain under a purified and characterizable form defined products by reference to the product according to **VITIELLO** and including, like this latter, two palmitoyl chains : both **VITIELLO**'s spacers are reproduced or modified for one or for both spacers according to the present invention.

In lipopeptide no. 1, such sequences are identical to those of the lipopeptide disclosed by **VITIELLO et al.** Two serines are inserted between the lipid portion and the auxiliary epitope. Three alanines, for their part, are inserted between the auxiliary epitope and the CTL epitope.

The lipopeptide no. 2 comprises the SS sequences (seryl-seryl) and **ARG-GLY-ARG**.

The lipopeptide no. 3 is built according to the present invention and comprises hydrophilic spacers respectively showing the **GLY-ARG** and **ARG-GLY-ARG** sequences.

The synthesis has been performed according to "standard" procedures used in solid phase according to the Boc-benzyl strategy disclosed by **Merrifield** (1963 J. Am. Chem. Soc. 85, 2149-2154; 1986 Science 232, 341-347). The introduction into N-terminal position of a di-Boc lysine allows, after deprotection of the peptidyl resin, for the simultaneous introduction of both palmitoyl chains so as to obtain the expected derivative.

2. Purification and physico-chemical properties of the lipopeptides

The lipopeptide no. 1 is practically insoluble in water or in a 10% DMSO/water mixture V/V. It proves to be non purifiable, non HPLC

characterizable according to resolving methods due to aggregate formation. It has been possible to confirm the identity of this lipopeptide by measuring the mass using PDMS-TOF spectrometry, performed on the rough product, immediately after the terminal cleavage by the hydrofluoric acid and before freeze-drying. After freeze-drying the product is wholly insoluble in water.

Mass measurement using PDMS-TOF spectrometry of the lipopeptide no. 2 is possible and confirms that the expected product has indeed been obtained. However, this product shows in water a behaviour close to that of the lipopeptide no. 1 and thus cannot be properly analyzed.

The lipopeptide no. 3 is soluble in 80% acetic acid. It can be sterilized through filtration on a 0.22 µm microfilter. It may be further dissolved in a 10% DMSO/water solution V/V. Mass measurement using PDMS-TOF spectrometry confirms the presence of the expected product. The product proves to be purifiable after injection of a concentrated solution into the DMSO, on a C4 Vydac column, at 60°C using an acetonitrile gradient in the presence of the TFA counter-ion. The product is characterizable by HPLC according to several conventional resolving methods, on C4 Vydac, C3 Zorbax, CN Zorbax and C1 Zorbax columns at 60°C using acetonitrile gradients in the presence of a TFA counter-ion or using an organic modifier such as isopropanol or butanol in isocratics.

The trifluoroacetate of the lipopeptide no. 3 is soluble in DMSO (20-25 mg/ml) and remains soluble after water dilution (10% DMSO). Substituting the trifluoroacetate counter-ion for an acetate counter-ion can be done by RP-HPLC on a C4 column : the product dissolved in 80% DMSO is injected onto the column equilibrated by solvent A (5% acetic acid in water). After removal of the non retained products (TFA counter-ions, salts), the product is eluted by means of a gradient from the solvent A to the solvent B (80% acetonitrile - 5% acetic acid – 15% water).

The acetate shows a solubility comparable to that of the trifluoroacetate. It has been used to immunize transgenic mice expressing for HLA-A2 and has proved to be able to induce a satisfactory CTL response in the absence of an immunization medium.

These results therefore show that inserting a hydrophilic spacer between the lipid portion and the auxiliary epitope on the one hand, and on the other hand, between the auxiliary epitope and the CTL epitope makes it

possible to solubilize the lipopeptide, which is not the case when the amino-acid sequences are different.

Exemple 2 : Synthesis of monopalmitoyled lipopeptides according to the invention and physico-chemical properties

A second series of lipopeptides has been produced in order to assess the possibility to obtain under a purified and characterizable form products comprising this time a single palmitoyl chain; the central spacer being used is introduced according to the present invention (-RGR- for arginyl-glycyl-arginyl-), whereas the **VITIELLO**'s -SS-(seryl-seryl) spacer is maintained or substituted for one or both spacers according to the present invention.

The introduction in N-terminal position of an alpha-Fmoc, epsilon Boc lysine allows, after a selective deprotection of the Boc group, for the introduction of a single palmitoyl chain in order to obtain the following product :

Lipopeptide no. 4

H-K(Pam)-SS-QYIKANSKFIGITE-RGR-AAGIGILTV

This product shows to be hardly purifiable. It is only soluble in an aqueous medium in the presence of DMSO. A chromatographic profile may be obtained by RP-HPLC only on a C1 type column using a "standard" solvent system (acetonitrile/water/trifluoroacetic acid) according to a hardly resolving method that does not allow to ensure the true purity of the product.

The following lipopeptide no. 5 has been synthetized.

Lipopeptide no. 5

H-K(Pam)-GR-QYIKANSKFIGITE-RGR-AAGIGILTV

This product has been obtained as for the lipopeptide no. 4 by a selective acylation of the epsilon NH_2 function of the N-terminal lysine.

This lipopeptide no. 5 meets the conventional purification and characterization standard.

This comparison between lipopeptides nos. 4 and 5, which are monopalmitoyl lipopeptides, confirms the results obtained with the dipalmitoyl lipopeptides.

The lipopeptides nos. 3 and 5 have been the only ones to meet the defined standards (access to a purification method and to resolving

analytical standards). Their study has been pursued with the study of their recognition by several cell types. The results of this study are shown in example 3.

Example 3: Biological activity of dipalmitoyl and monopalmitoyl lipopeptides according to the invention including a melanoma CTL epitope

The dipalmitoyl lipopeptide no. 3 and the monopalmitoyl lipopeptide no. 5 being synthetized as indicated respectively in examples 1 and 2 have been tested.

1. Study of the recognition of mono- and dipalm-27-35 Mart lipopeptides by a lineage of specific human cytotoxic T lymphocytes of 27-35 MART in a $_{\gamma}$-interferon (IFN-$_{\gamma}$) Elispot test

a) Materials and methods :

The L28.3 lineage of the specific cytotoxic T lymphocytes of 27-35 MART has been obtained from mononucleated cells of a LHA-$A2^{+}$ healthy donor's peripheral blood (PBMC). The induction protocole of such effector cells from naive donor's PBMC has been disclosed by **Ostankovitch et al.** (Int. J. Cancer, 1997, 72, 987-994).

Elispot test method

In this test, the target cells are non stimulated (naive) autologous PBMCs.

50 µl/well of human anti-IFN_{γ} murine antibody diluted in PBS buffer at a 4 µg/ml concentration are incubated in 96 well plates with nitrocellulose bottom overnight at 4°C in a humid chamber.

The PBMCs are unfrozen, maintained for 1 hour in RPMI medium containing 10% of AB (SAB) human serum at 37°C in an atmosphere containing 5% CO_2. They are subsequently incubated overnight with the peptides to be tested at different concentrations (10.5 and 1 µg/ml).

The wells are washed with PBS and subsequently saturated with RPMI medium containing 10% SAB, for 2 hours at 37°C.

The effector cells are then distributed in the 96 well plates, previously incubated with the anti-IFN_{γ} in quantity of 20,000 cells per well in final 200 µl and in the presence of stimulated autologous PBMCs with an effector:target (E:T) ratio of 1:1. The 4 µg/ml concentration phytohaemagglutinin (PHA) and the phorbol 12-meristate 13-acetate

(PMA)-ionomycin (150 and 500 ng/ml respectively) are used as positive controls.

After 20 hour incubation at 37°C in an atmosphere containing 5% CO_2, the wells are washed 5 times with PBS and then one time with distilled water and are then incubated overnight at 4°C with 100 µl/well of human anti-IFN_γ rabbit antibody diluted at 1/250 in RPMI containing 10% SAB. The wells are then washed 5 times with PBS containing 0.05% Tween 20 and subsequently incubated for 2 hours at 37°C with 100 µl biotinylated rabbit anti-immunoglobulin goat antibody diluted at 1/500 in PBS containing 0.05% Tween 20 and 1% BSA (bovine albumine serum).

The wells are washed again five times with PBS-Tween and then incubated for 1 hour at 37°C with 100 µl ExtrAvidine-Alkaline Phosphatase diluted at 1/600 in 1% PBS-Tween-BSA.

After four washings with PBS-Tween, the wells are incubated with 100 µl revelation substrate (Alkaline phosphatase conjugate substrate kit. Ref. 170-6432 Biorad.). The wells are subsequently washed with distilled water and are dried before reading the results on the stereomicroscope.

b) Results:

Fig. 1 shows the results obtained.

Both 27-35 MART lipopeptides specifically stimulate IFN_γ secretion through the lineage of anti 27-35 MART CTLs. They are therefore presented by the PBMC's and recognized by the CTLs in the same way as the nominal 27-35 MART peptide.

2. Study of the recognition of mono- and dipalmoyl 27-35 Mart lipopeptides by two clones of infiltrating lymphocytes of specific human melanoma of 27-35 MART using a toxicity test by ^{51}Cr use

a) Materials and methods :

LT8 and LT12 are two HLA-$A2^+$ clones obtained by re-stimulating melanoma infiltrating lymphocytes (TILs), that specifically recognize the 27-35 Mart peptide.

The target cells being used in this test are HLA-$A2^+$ human cells of T2 type. Such cells are free of peptide carriers and therefore only present the exogenous peptides on their free molecules of class I.

In this test, the target cells are incubated for one night with the peptide to be tested at a rate of 4 µg/10^6 cells in an atmosphere containing

5% CO_2 at 37°C. They are subsequently incubated for one hour at 37°C with 100 µCi sodium chromate (^{51}Cr). The target cells are then washed twice in a physiological serum containing 5% of calf's fetal serum (SVF), taken up in a 5% SVF RPMI medium and distributed into 96 well plates, with 3000 to 5000 cells per well in 100 µl.

The effector cells (LT8 and LT12) are then added in 100 µl of the same medium with an effector:target ratio of 10:1.

The chromium content obtained during the 4 hour incubation at 37°C has been measured on a gamma counter. The lysis percentage is determined by the following formula (R = content) :

% lysis (experimental R.-spontaneous R./total R.-spontaneous R.) x 100.

b) Results :

Figs. 2A and 2B illustrate the obtained results.

Both mono- and dipalmitic 27-35 MART lipopeptides are recognized by human effector cells, issued from melanomas and specific of the 27-35 MART peptide. These two lipopeptide types are recognized similarly and at a level at least comparable to the original peptide (27-35 MART), since the target cells have been exposed at the same final peptide concentration, whereas the lipopeptide molar weight is about 5 times higher than that of the 27-35 MART peptide.

3. Characterization of the "Helper" effect of the TT 830-843 auxiliary T epitope

a) Materials and methods :

The A2/Kb transgenic mice (3 to 4 in each group) have been IFA immunized by a subcutaneous route at the tail base and in the plantar pads with either (A) physiological serum or (B) 200 µg Mp-MART or (C) 200 µg Dp-MART. The mice were sacrificed 11 days later.

A proliferation test of the draining gland cells has been carried out with a three day culture in the presence of the TT 830-843 peptide (10 µg/ml) followed by 18 hours of addition of tritiated thymidine.

The supernatant has been collected after 36 hour culture for the IFN-γ dosage using the ELISA method.

b) Results :

In order to define the induction capacity of an auxiliary T response brought by the lipopeptide structures according to the invention, the

interferon-γ secretion as well as the lymphocyte proliferation in response to the TT 830-843 auxiliary T epitope have been studied in mice immunized with Mp-MART or Dp-MART in the presence of Freund's incomplete medium.

The results are reported in Fig. 3.

The results indicate that the Mp-MART structure and the Dp-MART structure (B, C) both induce a high interferon-γ secretion through the lymphatic gland cells of transgenic mice of A2/K^b type.

Similarly, the Mp-MART and Dp-MART (B, C) lipopeptides generate a large lymphocyte proliferation in response to the TT 830-843 peptide.

These results show that the TT 830-843 auxiliary T epitope is able to induce an auxiliary T response in A2/K^b transgenic mice. Moreover, these results also show that the covalent bond of the TT 830-843 epitope within the lipopeptide structure does not reduce its ability to be presented to T cells.

4. Study of the medium effect of the lipid portion of the 27-35 MART lipopeptide structures

a) Materials and methods :

The A2/Kb transgenic mice (3 to 4 in each group) have been immunized with no medium by a subcutaneous route at the tail base and in the plantar pads with either (A) a mixture of 27-35 MART peptide (50 µg) and TT 830-843 (140 µg), or (B) 200 µg Mp-MART (in order to provide the equimolarity of the 27-35 MART peptide against A), or (C) 200 µg Dp-MART. The mices were sacrificed 11 days later.

The number of $CD8^+$ T cells specific of the 27-35 MART has been determined using an IFN-γ ELISPOT test, wherein the cells are being exposed to presenting cells of the A2/Kb Jurkat type, either non filled as a negative control, or filled with the 27-35 MART peptide (30 µg/ml). The results are expressed in number of IFN-γ secreting cells by million of cells being tested.

b) Results :

In order to optimise the co-linear structures of the lipopeptides comprising the cytotoxic 27-35 MART T epitope for human vaccination,

the ability of such lipopeptide structures to induce immunization in the absence of an exogenous medium has been tested.

The results are shown in Fig. 4.

More particularly, the immunogenicity of both lipopeptide co-linear structures comprising respectively one or two palmitoyl chains has been tested. The mice have been subjected to a single injection in the absence of a medium, with each of the lipopeptides or otherwise with the mixture of 27-35 MART epitope and TT 830-843 used as a control, and then the interferon-$_\gamma$ secretion by the splenocytes has been measured after an *in vitro* re-stimulation with the 27-35 MART peptide.

The results of Fig. 4 indicate that the Mp-MART and Dp-MART co-linear structures are both able to induce in vivo a $CD8^+$ cytotoxic cellular response specific of the cytotoxic 27-35 MART T epitope in the absence of any exogenous medium.

Similar results have been obtained with lymphatic gland cells.

These results show that the medium effect of the co-linear lipopeptide structures according to the invention is enough to induce a specific immunization against the cytotoxic T epitope, in the absence of any other medium compound.

Example 4 : Preparation of a lipopeptide comprising a hydrazone bond

The hydrazinopeptide and the aldehyde peptide have the following formulae :

hydrazinopeptide : $K(NH_2)$ILKEPVHGV-OH/epitope MHCl-POL HIV-1

aldehyde peptide : CHOCO-RTPPAYRPPNAPILK(Pam)-NH_2/epitope MHCII-HBVc

The aldehyde peptide comprises the 128-140 epitope of the hepatitis B virus core protein (HBVc) such as disclosed by **MILICH et al.** (1998, Proc. Natl. Acad. Sci. USA, 85, 1610-1614).

16 mg (12 µmol) hydrazinopeptide and 15 mg (7 µmol) aldehyde peptide are dissolved in 4 ml 0.01 M citrate/phosphate buffer at pH 5.4 and 1 ml DMSO. pH is adjusted at 5.4 with 0.2 M Na_2HPO_4.

After 24 hours, the medium is diluted with 5 ml acetic acid and purified on a C18 column (15 x 500 mm). Eluent A : TFA 0.05% in H_2O, Eluent B : TFA 0.05% in CH_3CN/H_2O (80/20). Gradient 0-40% B in

10 min, then 40-100% B in 60 min. The pure fractions are collected and dry-frozen. 4.9 mg pure product is obtained (yield 22%).

The final product formula is represented on Fig. 5.

Exemple 5 : Biological activity of the lipopeptide structures according to the invention containing different HIV epitopes

a) Materials and Methods :

1. Peptide synthesis and characterization

Lipopeptides have been synthetized on a MBHA resin (Applied Biosystems, Foster City, USA) using the conventional BOC-benzyl strategy (Merrifield, RB, 1986, Science : Volume 232, pages 341-343; Merrifield RB, 1963, J. Am. Chem. Soc. Vol. 85:2149-2154) and 0.33 M 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in N-methyl-pyrrolidinone (NMP) with an in situ neutralization by diisopropylethylamine (DIPEA) (Schnolzer et al., 1992, Int. J. Pept. Protein. Res. vol. 40, pages 180-193) in a peptide automatic synthetizer of the Applied Biosystem 430A type (Foster City, USA). A double link has been systematically carried out using four protected amino-acid equivalents, followed by a systematic acetylation step using acetic anhydride.

For the synthesis of dipalmitoyl peptides, a Boc-Lys(Boc)-OH group (Senn Chemicals AG, Dielsdorf, CH) has been incorporated at the N-terminal end : the Boc groups have been simultaneously removed by a conventional TFA treatment. For the synthesis of monopalmitoyl peptides, a Boc-Lys(Fmoc)-OH group (Senn Chemicals AG, Dielsdorf, CH) has been incorporated at the N-terminal end, which offered the possibility of a selective removal of the Fmoc group by 20% piperidine in the NMP. In all cases, a palmitoylation step on resin has been carried out using a HBTU activation in the presence of DIEA, as above, on the incidentally fully protected peptide. The lipopeptides have then been deprotected and separated from the resin by a hydrogen fluoride treatment (Tam, J.P. 1985, Int. J. Pept. Protein Res, Volume 26, pages 262-273) in a Teflon-Kel F device (Asti, Courbevoie, France) : a "High HF" protocole has been used for the TT-POL1 and TT-GAG lipopeptides, a "Low-High HF" type protocole has been used for the TT-POL2 and TT-NEF lipopeptides. The lipopeptides have been partially purified upon a precipitation step by cold diethyl ether in 5 ml of a trifluoroacetic acid solution and subsequently dry-

frozen. The purifications have been carried out with several parallel RP-HPLC cycles : for each cycle, 50 to 100 mg rough lipopeptide have been dissolved in 2 ml DMSO and then diluted in 1 ml water, before injection on :

- for monopalmitoylpeptides, a 12.5 mm x 250 mm size column loaded with a stationary phase of C18 (0.03, 5 µm) Hyperprep Hypersil type eluted at 5 ml/min,
- for dipalmitoylpeptides, a 12.5 mm x 250 mm column loaded with Zorbax C3 (0, 03, 5 µm) and eluted at 4 ml/min.

The compounds have been eluted with a solvent system comprising acetonitrile/H_2O/0.05% TFA at 60°C. The homogeneity has been confirmed by means of a RP-HPLC on two different analytical columns (one C18 Vydac column of 4.6 x 250 and one C3 Zorbax column of 4.6 x 150 mm) : the lipopeptides were more than 90% pure. The peptide characterization has been checked by determining the amino-acid composition after a total hydrolysis and determination of the molecular weight by TOF-PDMS (plasma desorption mass spectrometer, Bio-Ion 20, Uppsala, Suède). The measured molecular weights have been as follows : monopalmitoyl derivatives TT-GAG :$[M+H]^+$ calc. 3534, obs. 3523; TT-NEF:$[M+H]^+$ calc. 4317, obs. 4318, TT-POL1:$[M+H]^+$ calc. 3534, obs. 3535; TT-POL2:$[M+H]^+$ calc. 4142, obs. 4143. For dipalmitoyl derivatives : TT-GAG:$[M+H]^+$ calc. 3763, obs. 3762; TT-NEF:$[M+H]^+$ calc. 4556, obs. 4553, TT-POL1:$[M+H]^+$ calc. 3773, obs. nd.; TT-POL2:$[M+H]^+$ calc. 4380, obs. 4378. All the compounds have been obtained in the form of a dry-frozen powder. The monopalmitoyl derivatives are water soluble. The dipalmitoyl derivatives are soluble in a water-DMSO mixture. The purification yields have been :

- for monopalmitoyl derivatives : TT-GAG : 16%, TT-NEF : 28%, TT-POL1 : 33%, TT-POL2 : 12%,
- for dipalmitoyl derivatives : TT-GAG : 16%, TT-NEF : 39%, TT-POL1: 22%, TT-POL2 : 4.5%.

The amino-acid sequences of the peptides and lipopeptides are shown in table 8.

2. Mice and immunization

HLA-A2 transgenic mice have been used expressing the α1 and α2 domains of the HLA-A2 molecule (Vitiello et al., 1991, J. Exp. Med. Vol. 173, pages 1007-1015). The α3 field of the heavy chain corresponds to the H-2 K^b murine domain. This feature enables the $CD8^+$ molecule expressed by the $CD8^+$ murine T cells to interact with the syngenetic α3 domain of the hybrid MHC molecule of class I.

HLA-A3 transgenic mice have also been used.

All the peptides and lipopeptides have been dissolved in a saline solution in the presence of 10% DMSO. The induction of cytotoxic T cells in HLA transgenic mice has been performed as disclosed by SETTE et al., (1994) (J. Immunol. vol. 153, pages 5586-5592).

Then, CTL epitopes (50 µmg/mouse) have been mixed with TT 830-843 auxiliary T epitope (140 µm/mouse), emulsified in Freund's incomplete medium (IFA) and injected at the tail base. The lipopeptides have been injected alone (150 µm/mouse), diluted in a saline solution with no Freund's incomplete medium (IFA). Eleven days after the injection, the mice have been sacrificed and the splenocytes as well as the lymphatic gland cells (LNC) have been incubated at a rate of 3×10^6 per ml in a complete medium in the presence of irradiated syngenetic lymphoblasts stimulated by LPS and covered with the peptides being used as stimulating cells. The complete medium comprises 1640 RPMI medium (Gibco/BRL, Gaithersburg, MD), 10% SVF, 2 mM glutamine, antibiotics and β2-mercaptoethanol (5×10^{-5}M). After six days, the splenocytes and the lymphatic gland cells have been tested for interferon-γ secretion according to the ELISPOT technique described in example 3 for mouse cells and the ELISPOT technique described thereafter for the human PBMCs.

3. ELISPOT test on human PBMCs

HIV seropositive donors have been selected for their class I HLA molecule expression according to data well known to the man skilled in the art in the field of restriction to peptides and lipopeptides.

Nitrocellulose microplates (Millipore, Bedford, MA) have been incubated overnight at 4°C with 1 µg/ml of a human anti-interferon-γ capture antibody diluted in a carbonate/bicarbonate buffer at pH 9.6 (100 µl/well). After 5 washing cycles, the plates have been saturated for

2 hours at 37°C with 200 µl, followed by washing. For the human cell cultures, the complete medium was made of 1640 RPMI, 10% bovine albumine serum, 2 mM glutamine, antibiotics and β2-mercaptoethanol (5 x 10^{-5} M). For the peptide test, patients' PBMCs have been added directly to the nitrocellulose microplates (2 x 10^5 cells/well) and incubated for 24 hours at 37°C in the presence of 10 mg/ml of the peptide. For the lipopeptide test, the PBMCs have been incubated overnight at 37°C with the lipopeptides and then washed and irradiated (3000 rads). These cells have been used as stimulating cells in a co-culture (2 x 10^5 cells/well) with syngenetic PBMCs (ratio : 1/1) in nitrocellulose plates for 24 hours at 37°C. After 24 hours, the plates containing the direct culture (peptide) or the co-culture (lipopeptide) have been washed and incubated for 2 hours at room temperature in the presence of 100 µl of a biotinylated mouse's anti-interferon-$_\gamma$ monoclonal antibody diluted at 1 µg/ml in a PBS-Tween buffer (0.05%) and bovine albumine serum (1%). After five washing cycles in 0.05% PBS-Tween, the plates have been incubated for one hour at room temperature in the presence of extravidine marked with alkaline phosphatase (Sigma, Germany) diluted at 1/5000 in a PBS-Tween buffer (0.05%) and of calf's fœtal serum (1% SVF). The "spots" have been revealed by adding a chromogenic conjugated substrate of the alkaline phosphatase (Biorad, Hercules, CA) according to the manufacturer's instructions and the positive spot forming cells for the interferon-$_\gamma$ have been counted with a binocular magnifying glass.

4. Proliferation test

The lymphatic gland cells and the extracted splenocytes have been cultivated in 96 well microplates at a rate of 5.10^5 cells/well in a final volume of 200 µl for the complete medium. The cells have been stimulated or not with 20 µg/ml of a CD4 epitope limited to the MHC molecules of class II, the TT 830-843 epitope. After a 72 hour culture at 37°C, the cells have been incubated overnight with 0.25 µCi [^{3}H] thymidine (Amersham, Life Technology). The cells have been collected and the [^{3}H] thymidine addition has been analyzed in a "Microbetaplate" device (Wallac, Turku, Finland).

5. Cells and monoclonal antibodies

Jurkat-A2 (J A2/K^b) cells, a lineage of human leukemic T cells transfected with a plasmid coding for the hybrid HLA-A2/$H2K^b$ molecule (Irvin et al. 1989).

The lymphoblasts used to stimulate the effector cells *in vitro* have been prepared as follows : HLA-A2 transgenic mouse splenocytes have been cultivated with 25 µg/ml LPS (E. Coli) and 7 µg/ml Dextran sulfate (Sigma). After 72 hours at 37°C, the cells have been washed and incubated with the CD8 epitope for 2 hours at 37°C. The cells have then been irradiated (3000 rads), washed three times and co-cultivated with the effector cells.

The mouse anti-interferon-γ capture and detection monoclonal antibodies (R4-6A2 and XMG1.2 clones, respectively) are marketed by Pharmingen (San Diego, USA). The human anti-interferon-γ capture and detection monoclonal antibodies (1-D1K and 7-B6-1-biotin clones, respectively) are marketed by Mabtech (Stockholm, Sweden).

b) RESULTS

1) Peptide and lipopeptide antigenicity in man

The capacity of the selected peptides including the epitopes of interest for efficiently stimulating the specific cytotoxic T lymphocytes of HIV seropositive donors has been tested. The activation of the epitope specific CTLs has been followed by measuring the interferon-γ secretion after incubation of patients' PBMC's with the peptides, using the ELISPOT test. When it has been found that the peptides efficiently stimulated the specific CTLs, the activation capacity of these epitope specific CTLs has been studied with the corresponding lipopeptides pre-incubated with the syngenetic PBMC's.

The results illustrated in Fig. 6a show that the CTLs for the Z77 patient (of HLA-2 type; positive for HIV) recognize the POL 476-484 and GAG 77-85 peptides. The TT-POL1 and TT-GAG lipopeptides have also strongly stimulated these specific CTLs. No significant antigenicity difference has been found between the monopalmitoyl and the dipalmitoyl structures for these two lipopeptides. Moreover, no activation of the CTLs specific for 346-354 POL in the Z77 patient's PBMC's, either with the 346-354 POL peptide or with the corresponding TT-POL2 lipopeptides

(monopalmitoyl and dipalmitoyl structures). However, as indicated in Fig. 6a, the HLA-A2 Z108 patient's CTLs hardly recognize the 346-354 epitope and the TT-POL2 lipopeptides more significantly.

The results in Fig. 6b show that the 73-82 NEF peptide, an epitope binding with both HLA-A11 and HLA-A3 molecules and forming stable complexes with these molecules, also induces a strong specific CTL response in HLA-A11 and HLA-A3 patients' (respectively Z 129 and P48) PBMC's. Moreover, the TT-NEF monopalmitoyl lipopeptide has also induced a strong CTL response from these PBMCs.

Finally, the results in Fig. 6c show that all the peptides selected for their binding capacity on HLA-B35 purified molecules as well as for their capacity to form stable HLA/peptide complexes (see Fig. 6c), were very antigenic in the HLA-B35 (P3) patient. The TT-NEF monopalmitoyl and dipalmitoyl lipopeptides, including the NEF 74-81, NEF 71-79 and NEF 68-76 peptides, were also antigenic with the P3 patient.

However, the TT-POL2 (monopalmitoyl and dipalmitoyl) lipopeptides, although containing the well recognized POL-342-350 epitope, were not antigenic with the HLA-B35 patients.

The results in Fig. 6c also indicate that the NEF 68-76 peptide, a potential HLA-B7 epitope, has efficiently induced a specific CTL response in the HLA-B7 Z118 patient's PBMCs. Moreover, the TT-NEF lipopeptides were also antigenic in the HLA-B7 patients.

It is noteworthy to emphasize that the results of figures 6b and 6c show that a multiepitope lipopeptide can be efficiently recognized by individuals expressing different HLA molecules of class I.

This set of results clearly shows that the lipopeptides can be efficiently transformed by the PBMCs and can activate the specific CTLs of epitopes.

2. Study of the peptide and lipopeptide immunogenicity in HLA-A2 and HLA-A3 transgenic mice

The aim of this study was to determine whether the lipopeptides were able to induce the production of specific CTL in naive organisms, since they have never been sensitized with a HIV epitope and do not possess any pre-existing epitope specific CTL cell.

The lipopeptides and peptides comprising the epitopes of interest have been injected in transgenic mice expressing for HLA-A2 or HLA-A3 molecules. The cytotoxic T epitope carrier peptides have been co-injected with the TT 830-843 auxiliary T epitope and emulsified in Freund's incomplete medium.

The lipopeptides have been diluted in a saline solution and injected without Freund's incomplete medium. The production of epitope specific CTL cells in mice has been followed as indicated in the Materials and Methods chapter.

The results in Fig. 7a show that the three epitopes limited to HLA-A2, respectively GAG 77-85, POL 476-484 and POL 346-354, induced a low specific CTL response in HLA-A2 transgenic mice, but that the corresponding monopalmitoyl lipopeptides were able to induce a higher CTL response. In all cases, the dipalmitoyl lipopeptides induced a weaker CTL response than the monopalmitoyl lipopeptides.

The results in Fig. 7b indicate that the NEF-73-82 epitope induces a strong specific CTL response in HLA-A3 transgenic mice. Moreover, the TT-NEF monopalmitoyl lipopeptides were able to induce a CTL response as high as NEF 73-82 epitope.

3. Study of the need for an auxiliary T type response for inducing CTL cells

In order to determine the amount of the auxiliary T effect in the production of CTL cells by the lipopeptides according to the invention, HIV epitope containing monopalmitoyl lipopeptides, but free from TT 831-843 epitope, have been built.

The results in Fig. 8a show that the auxiliary T epitope-free POL1 lipopeptide does not induce any CTL specific response, whereas the TT-POL1 lipopeptide was able to induce a much higher specific CTL response. This result indicates that the auxiliary T peptide is necessary to induce a high CTL response when the lipopeptides are injected at the base of the HLA-A2 transgenic mice tail. Lymphocyte proliferation experiments have been carried out in order to determine whether the TT 831-843 auxiliary T epitope, contained in the lipopeptides, was able to induce efficiently an auxiliary T response.

The results presented in Fig. 8b show that the lymphatic gland cells of mice immunized with the TT-POL 1 monopalmitoyl lipopeptide proliferated in response to TT 380-843 peptides as strongly as the lymphatic gland cells of mice immunized with the TT 830-843 auxiliary T epitope emulsified in Freund's incomplete medium.

These results show that a response of the auxiliary T type is necessary to obtain a high specific CTL response and that the auxiliary T response is induced by the auxiliary T epitope contained in the lipopeptidic co-linear structures.

TABLE 1 : BCR-ABL epitopes

Peptide	Sequence	Binding with HLA
247-255	EDAELNPRF	B44
488-496	SELDLEKGL	B44
768-776	DELEAVPNI	B44
901-934 b2a2	KEDALQRPV	B44
902-935 b2a2	EDALQRPVA	B44
986-994	GEKLRVLGY	B44
1176-1184	EDTMEVEEF	B44
1252-1260	MEYLEKKNF	B44
1691-1699	NEEAADEVF	B44
49-57	VNQERFRMI	B8
580-588	LFQKLASQL	B8
722-730	ARKLRHVFL	B8
786-794	ALKIKISQI	B8
886-893	CVKLQTVH	B8
928-936 b3a2	KALQRPVAS	B8
1830-1838	GAKTKATSL	B8
1975-1983	IQQMRNKFA	B8
1977-1984	QMRNKFAF	B8
252-260	NPRFLKDNL	B7
329-338	TPDCSSNENL	B7
693-701	TPRRQSMTV	B7
1058-1066	SPGQRSISL	B7
1196-1205	HPNLVQLLGV	B7

1560-1569	SPKPSNGAGV	B7
1717-1725	KPLRRQVTV	B7
1878-1884	SPAPVPSTL	B7
36-44	ERCKASIRR	B27
71-79	DRQRWGFFRR	B27
575-583	QRVGDLFQK	B27
834-842	FRVHSRNGK	B27
642-650	LLYKPVDRV	A2
684-692	FLSSINEEI	A2
708-716	QLLKDSFMV	A2
714-722	FMVELVEGA	A2
817-825	KLSEQESLL	A2
881-889	MLTNSCVKL	A2
908-917	GLYGFLNVIV	A2
912-920	FLNVTVHSA	A2
1240-1248	VLLYMATQI	A2
1903-1911	FIPLISTRV	A2
1932-1940	VVLDSTEAL	A2
50-58	NQERFRMIY	A1
223-231	VGDASRPPY	A1
549-558	KVPELYEIHK	A3/A11
583-591	KLASQLGVY	A3/A11
715-724	MVELVEGARK	A3/A11
916-923	IVHSATGFK	A3/A11
620-928 b3a2	ATGFKQSSK	A3/A11
924-932 b3a2	KQSSKALQR	A3/A11
1156-1165	EVYEGVWKKY	A3/A11
1311-1320	SLAYNKFSIK	A3/A11
1499-1509	NLFSALIKK	A3/A11
1724-1734	TVAPASGLPHK	A3/A11
1905-1914	LISTRVSLRK	A3/A11
1922-1930	RIASGAITK	A3/A11

TABLE 2 : p53 epitopes

- p53 epitopes binding with HLA-A1 :

RVEGNLARVEY (196-205)

GSDCTTIHY (226-234)

- p53 epitopes binding with HLA-A2 :

LLPENNVLSPL (25-35)

RMPEAAPPV (65-73)

RMPEAAPRV

ALNKMFCQL (129-137)

STPPPGTRV (149-157)

GLAPPQHLIRV (187-197)

LLGRNSFEV (264-272)

PLDGEYFTL (322-330)

- p53 epitopes binding with HLA-A3 :

RVRAMAIYK (156-164)

RRTEEENLR (282-290)

ELPPGSTKR (298-306)

- p53 epitopes binding with HLA-B7 :

LPENNVLSPL (26-35)

APRMPEAAPPV (63-73)

APRMPEAAPRV

APPQHLIRV (189-197)

RPILTIITL (249-257)

KPLDGETYFTL (321-330)

- p53 epitopes binding with HLA-B8 :

CQLAKTCPV (135-143)

GLAPPQHLI (187-195)

NTFRHSVVV (210-218)

- p53 epitopes binding with HLA-B51 :

LLPENNVLSPL (25-35)

RMPEAAPPV (65-73)

LIRVEGNLRV (194-203)

TABLE 3 – E_6 and E_7 protein epitopes

YMLDLQPETT (E7 11-20)
LLMGTLGIV (E7 82-90)
TLGIVCPI (E7 86-93)
TIHDIILECV (E6 29-38)
KLPQLCTEL (E6 18-26)
RPPKLPQL (E6 8-15)
LRREVYDFAFRDLCIVYRDGNPY (E6 45-67)
ISEYRHYCY (E6 80-88)
EKQRHLDKKQRFHNIRGRWT (E6 121-140)
GQAEPDRAHYNIVTF (E7 43-57)
QAEPDRAHY (E7 44-52)
EPDRAHYNIV (E7 46-55)

TABLE 4 : VIH-1 virus epitopes

HLA-A1

(Nef 96-106: GLEGLIHSQRR
(Nef 121-128: FPDWQNYT
(NEF 137-145: LTFGWCYKL
(Nef 184-191: RFDSRLAF
(Nef 195-202: ARELHPEY

HLA-A2

Gp120 121-129: KLTPLCVTL
P17 77-85: SLYNTVATL
RT 200-208: ALVEICTEM
RT 275-285: VLDVGDAYFSV
RT 346-354: KIYQYMDDL
RT 368-376: KIEELRQHL
RT 376-387: LLRWGLTTPDK
RT 476-484: ILKEPVHGV
RT 588-596: PLVKLWYQL
RT 683-692: ELVNQDEQL
Nef 136-145: PLTFGWCFKL
Nef 180-189: VLQWRFDSRL
Nef 190-198: ALHHVAREL

Gp41 818-826: SLLNATVDI
P24 185-193: DLNTMLNTV
RT 346-354: VIYQYMDDL
RT 588-596: PLVKLWYQL
Pro 143-152: VLVGPTPVNI
(Gp120 37-44: TVYYGVPV
(Gp120 115-122: SLKPCVKL
(Gp120 313-321: RIQRGPGRA
(Gp120 197-205: TLTSCNTSV
(Gp120 428-435: FINMWQEV
(Gp41 836-844: VVQGAYRAI
(p24 219-228: HAGPIAPGQM
(P15 422-431: QMKDCTERQA
(p15 448-456: FLQSRPETA
(RT 681-691: ESELVNQIIEG

HLA-A3

P17 18-26: KIRLRPGGK
P17 20 28:RLRPGGKKK
RT 200-210: ALVEICTEMEK
RT 325-333:AIFQSSMTK
RT 359-368: DLEIGQHRTK
Nef 73-82: QVPLRPMTYK
Gp120 37-46: TVYYGVPVWK
Gp41 775-785: RLRDLLLIVTR
P17 18-26: KIRLRPGGK

HLA-A11

RT 325-333: AIFQSSMTK
RT 507-517: QIYQEPFKNLK
Nef 73-82: QVPLRPMTYK
Nef 84-92: AVDLSHFLK
p24 349-359: ACQVGGPGHK
P17 83-91:ATLYCVHQR

HLA-A24 (A9)

Gp120 52-61: LFCASDAKAY
Gp41 591-598: YLKDQQLL

or 590-597: RYLKDQQLL
(RT 484-492: VYYDPSKDL
(RT 508-516: IYQEPFKNL
(RT 681-691: ESELVNQIIEG

HLA-A25 (A10)
P24 203-212: ETINEEAAEW

HLA-A26 (A10)
P24 167-175: EVIPMFSAL

HLA-A30 (A19)
(Gp41 845-852: RAIRHIPRR

HLA-A31 (A19)
Gp41 775-785: RLRDLLLIVTR

HLA-A32 (A19)
Gp120 424-432: RIKQIINMW
Gp41 774-785: HRLRDLLLI
RT 559-568: PIQKETWETW

HLA-A33 (A19)
(P24 266-275: IILGLNKIVR

HLA-B7
RT 699-707: YLAWVPAHK
Nef 68-77:FPVTQVPLR
Nef 128-137: TPGPGVRYPL
Gp120 303-312: RPNNNTRKSI
Gp41 848-856: IPRRIRQGL
RT 699-77: YLAWVPAHK

HLA-B8
Gp120 2-10: RVKEKYQHL
P17 24-32: GGKKKYKLK
Nef 90-97: FLKEKGGL =
P24 259-267: GEIYKRWII
Gp41 591-598: YLKDQQLL
(Gp41 849-856: PRRIRQGL
or 851-859: RIRQGLERIL
(P24 329-337: DCKTILKAL
(RT 185-193: GPKVKQWPL

(Nef 182-189: EWRFDSRL =

HLA-B14

Gp41 589-597: ERYLKDQQL
P24 298-306: DRFYKTLRA
(P24 183-191 ? : DLNTMLNTV
(p24 304-313: LRAEQASVQEV
(p24 305-313: RAEQASVQEV

HLA-B18

Nef 135-143: YPLTFGWCY
Nef 135-143: YPLTFGWCF

HLA-B27

P24 263-272: KRWIILGLNK
Nef 73-82: QVPLRPMTYK
Nef 134-141: RYPLTFGW
or 133-141: YPLTFGW
Gp41 589-597: ERYLKDQQL
(Gp41 791-800: GRRGWEALKY

HLA-B35

Gp120 78-86: DPNPQEVVL
Gp120 257-265: RPVVSTQLL
RT 285-294: VPLDKDFRKY
RT 323-331: SPAIFQSSM
RT 342-350: NPDIVIYQY (consensus clade B)
RT 460-468: IPLTEEAEL
RT 598-608: EPIVGAETFY
Nef 68-76: FPVRPQVPL
Nef 74-81: VPLRPMTY
Gp41 611-619: TAVPWNASW
Gp120 42-52: VPVWKEATTTL
P17 124-132: NSSQVSQNY (consensus clade B)
P24 254-262: PPIPVGEIY (consensus clade B)

HLA-B37

Nef 120-128: YFPDWQNYT

HLA-B44 (B12)

P24 178-186: SEGATPQDL

(P24 175-184: LESGATPQDL

HLA-B51 (B5)

gp-41 562-570: RAIEAQQHL
RT 200-208: ALVEICTEM
RT 209-217: EKEGKISKI
RT 295-302: TAFTIPSI

HLA-B52 (B5)

Nef 190-198: AFHHVAREL

HLA-B55 (B22)

Gp120 42-51: VPVWKEATTTL

HLA-B57 and B58 (B17)

P24 240-249: TSLTQEQIGW
Nef 116-125: HTQGYFPDWQ
or 116-124: HTQGYFPDW
Nef 120-128: YFPDWQN
(P24 147-155: ISPRTLNAW
(P24 164-172: FSPEVIPMF

HLA-Bw62 (B15)

P17 20-29: RLRPGGKKKY
P24 268-277: LGLNKIVRMY
RT 427-438 : LVGKLNWASQIY
Nef 84-91: AVDLSHFL
Nef 117-127: TQGYFPDWQNY

HLA-Cw4

gp120 380-388: SFNCGGEFF

HLA-Cw8

RT 663-672: VTDSQYALGI
P24 305-313: RAEQASQEV
Nef 82-91: KAALDLSHPL

HLA-Cw?

P24 308-316: QATQEVKNW

TABLE 5 – Human melanoma epitopes

Gene/protein	Restriction MHC	Peptide	Position of amino-acids
Tyrosinase	HLA-A2	MLLAVLYCL	1-9
	HLA-A2	YMNGTMSQV	369-377
		YMDGTMSQV	
	HLA-A24	AFLPWHRLF	206-214
	HLA-B44	SEIWRDIDF	192-200
Pmel17^{gp100}	HLA-A2	KTWGQYWQV	154-162
	HLA-A2	AMLGTHTMEV	177-186
	HLA-A2	MLGTHTMEV	178-186
	HLA-A2	ITDQVPFSV	209-217
	HLA-A2	YLEPGPVTA	280-288
	HLA-A2	LLDGTATLRL	457-466
	HLA-A2	VLYRYGSFSV	476-485
	HLA-A2	SLADTNSLAV	570-579
	HLA-A3	ALLAVGATK	17-25
Melan-A^{MART-1}	HLA-A2	(E)AAGIGILTV	26(7)-35
	HLA-A2	ILTVILGVL	32-40
Gp$^{75TRP-1}$	HLA-A31	MSLQRQFLR	
TRP-2	HLA-A31	LLGPGRPYR	197-205

TABLE 6 – Tumor epitopes resulting from mutations

Gene/protein	Tumor	Restriction MHC	Peptide	Position of amino-acids
MUM-1	Melanome	HLA-B44	EEKLIVVLF	30-38
CDK4	Melanome	HLA-A2	ACDPHSGHFV	23-32
β-catenin	Melanome	HLA-A24	SYLDSGIHF	29-37
CASP-8	Squamous head and neck carcinome	HLA-B35	FPSDSWCYF	476-484

TABLE 7 – Antigens common to various tumors

Gene	Tissue where normal expression takes place	Restriction MHC	Antigenic peptide	Position of amino-acids
MAGE-1	Testicles	HLA-A1	EADPTGHSY	161-169
		HLA-Cw16	SAYGEPRKL	230-238
MAGE-3	Testicles	HLA-A1	EVDPIGHLY	168-176
		HLA-A2	FLWGPRALV	271-279
		HLA-B44	MEVDPIGHLY	167-176
BAGE	Testicles	HLA-Cw16	AARAVFLAL	2-10
GAGE-1/2	Testicles	HLA-Cw6	YRPRPRRY	9-16
RAGE-1	Retina	HLA-B7	SPSSNRIRNT	11-20
GnTV	None	HLA-A2	VLPDVFIRC	38-64

FOHOHO 99E2960

TABLE 8 : USED PEPTIDES AND LIPOPEPTIDES

Designation	Position	Amino-acid sequences	Restriction MHC-I	Reference
Peptides HIV				
GAG	77-85	SLYNTVATL	HLA-A2.01	(6)
POL	476-484	ILKEPVHGV	HLA-A2.01	(5)
POL	346-354	VIYQYMDDL	HLA-A2.01	(2)
POL	342-350	NPDIVIYQY	HLA-B35	(4)
POL	343-350	PDIVIYQY	HLA-B44	non described
POL	347-354	FYQYMDDL	HLA-A24	non described
POL	345-354	IVIYQYMDDL	HLA-A2	non described
NEF	68-76	FPVTPQVPL	HLA-B7-B35	(3)
NEF	71-79	TPQVPLRPM*	HLA-B35	non described
NEF	74-81	VPLRPMTY	HLA-B35	(1)
NEF	73-82	QVPLRPMTYK	HLA-A3-A11	(1)
Lipopeptides HIV				
TT-GAG	77-85 Palm-K-GR QYIKANSKFIGITE RGR SLYNTVATL		HLA-A2.01	
TT-POL1	476-484 Palm-K-GR QYIKANSKFIGITE RGR ILKEPVHGV		HLA-A2.01	
TT-POL2	Palm-K-GR QYIKANSKFIGITE RGR NPDI VIYQYMDDL multi-epitope		Multi HLA	
TT-NEF	Palm-K-GR QYIKANSKFIGITE RGR FPVTP QVPLRPMTYK multi-epitope		Multi HLA	
Auxiliary T peptide				
Tetanic toxine	830-843	QYIKANSKFIGITE	HLA-DR, H-2 K^b	

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o **HARRER, E., T. HARRER, P. BARBOSA, M. FEINBERG, R.P. JOHNSON, S. BUCHBINDER, and B.D. WALKER. 1996.** Recognition of the highly conserved YMDD region in the human immunodeficiency virus type 1 reverse transcriptase by HLA-A2- restricted cytotoxic T lymphocytes from an asymptomatic long-term nonprogressor. J. Infect Dis. 173:p476-9.

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o **TSOMIDES, T.J. B.D. WALKER, and H.N. EISEN. 1991**. An optimal viral peptide recognized by $CD8^+$ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein. Proc. Natl Acad. Sci USA. 88:p11276-80.

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09/673166
529 Rec'd PCT/PTO 06 OCT 2000

MODIFIED CLAIMS

1. A lipopeptide comprising at least one auxiliary T epitope, at least one CTL epitope and at least one lipid moiety characterized in that the epitopes and the lipid portion on the one hand and the epitopes on the other hand are independently separated by amino-acid sequences, so-called spacers, comprising linkages from 1 to 10 generally charged amino-acids in a neutral medium, providing the lipopeptide hydrophilic.

2. A lipopeptide according to claim 1, characterized in that at least one of the spacers comprises from 1 to 10 glycines and/or arginines.

3. A lipopeptide according to one of claims 1 to 2, characterized in that at least one of the spacers has one of the following sequences :

GLY ARG or

ARG GLY ARG

4. A lipopeptide according to one of claims 1 to 3, characterized in that at least one of the spacers comprises one or more glutamic and/or aspartic acids.

5. A lipopeptide according to one of claims 1 to 3, characterized in that the spacers comprise a cysteine or an alkyl chain functionalized by a thiol group and non peptide bonds.

6. A lipopeptide according to any one of claims 1 to 3, characterized in that the spacers comprise a group selected amongst a thiazolidine, oxime or hydrazone group.

7. A lipopeptide according to one of claims 1 to 6, characterized in that it comprises the linkage of :

- a lipid portion,
- a first spacer,
- an auxiliary T epitope,
- a second spacer, and
- a CTL epitope.

8. A lipopeptide according to one of claims 1 to 6, characterized in that it comprises the linkage of :

- a lipid portion,
- a first spacer,
- an auxiliary T epitope,

- a second spacer,
- a first CTL epitope,
- a third spacer, and
- a second CTL epitope.

9. A lipopeptide according to one of claims 1 to 8, characterized in that the lipid portion comprises one or more linear or branched, saturated or insaturated chains derived from C_{10}-C_{20} fatty acids or alcohols, or a steroid derivative.

10. A lipopeptide according to one of claims 1 to 9, characterized in that the lipid portion comprises at least two chains derived from C_{10}-C_{20} fatty acids or alcohols, bonded together by one or more amino-acids.

11. A lipopeptide according to one of claims 1 to 10, characterized in that the lipid portion is made of two palmitic acid chains linked to the NH_2 groups of a lysine.

12. A lipopeptide according to one of claims 1 to 10, characterized in that the lipid portion comprises one or more chains selected amongst the palmitic acid, the oleic acid, the linoleic acid, the linolenic acid, the 2-amino-hexadecanoic acid, the pimelautide or the trimexautide.

13. A lipopeptide according to one of claims 1 to 12, characterized in that the non lipid portion comprises from 15 to 100 amino-acids.

14. A lipopeptide according to one of claims 1 to 13, characterized in that the auxiliary T epitope is a multivalent epitope.

15. A lipopeptide according to one of claims 1 to 14, characterized in that the auxiliary T epitope is the 830-843 peptide of the tetanic toxin having the following sequence :

QYIKANSKFIGITE

16. A lipopeptide according to one of claims 1 to 14, characterized in that the auxiliary T epitope is the haemagglutinin epitope of the influenza virus or the PADRE epitope.

17. A lipopeptide according to one of claims 1 to 16, characterized in that it comprises at least a CTL epitope of a protein specific of the melanoma, a HIV protein, VBH, papillomavirus, protein p53 or a protein from the intrahepatocytary stadium of *Plasmodium falciparum*.

18. Use of a lipopeptide according to one of claims 1 to 17 for making a medicine or a vaccine for the induction of a specific immune

response.

19. Use of a lipopeptide according to one of claims 1 to 17 for making a medicine for the induction of an immune response against HIV, VBH, papillomavirus, the melanoma p-53 or malaria.

20. A pharmaceutical composition, characterized in that it comprises a pharmacologically efficient dose of one or more lipopeptides according to one of claims 1 to 17 and pharmaceutically compatible excipients.

21. A medicine or vaccine, characterized in that it comprises one or more lipopeptides according to one of claims 1 to 17.

0065765 A1

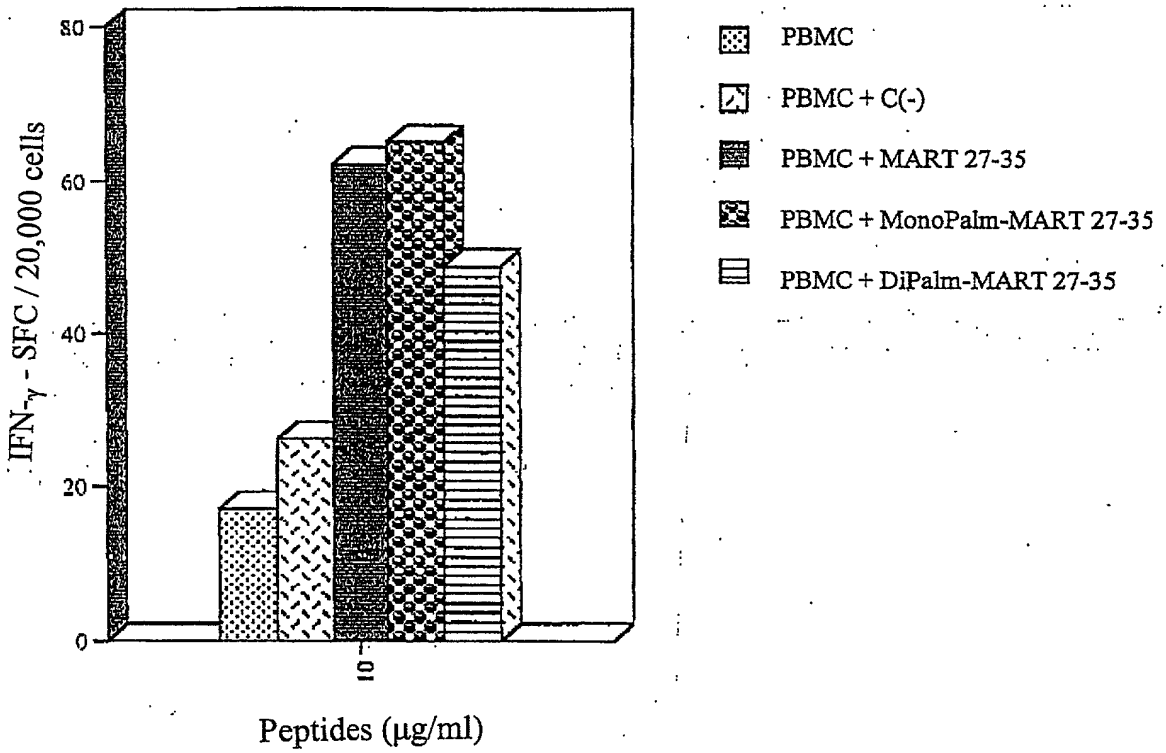

80
60
40
20
0
IFN-γ - SFC / 20,000 cells
10
Peptides (µg/ml)
PBMC
PBMC + C(-)
PBMC + MART 27-35
PBMC + MonoPalm-MART 27-35
PBMC + DiPalm-MART 27-35

FIGURE 1

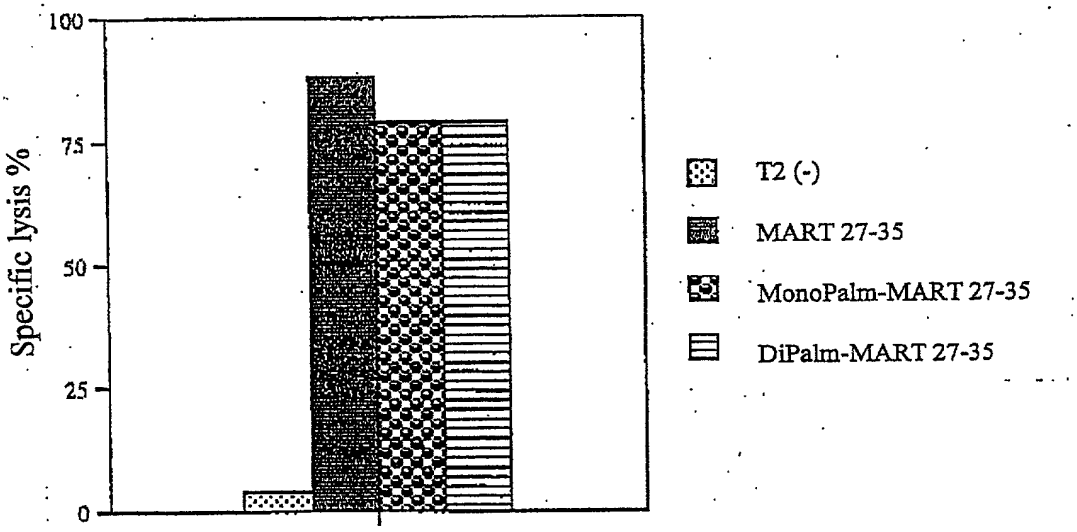


FIGURE 2A

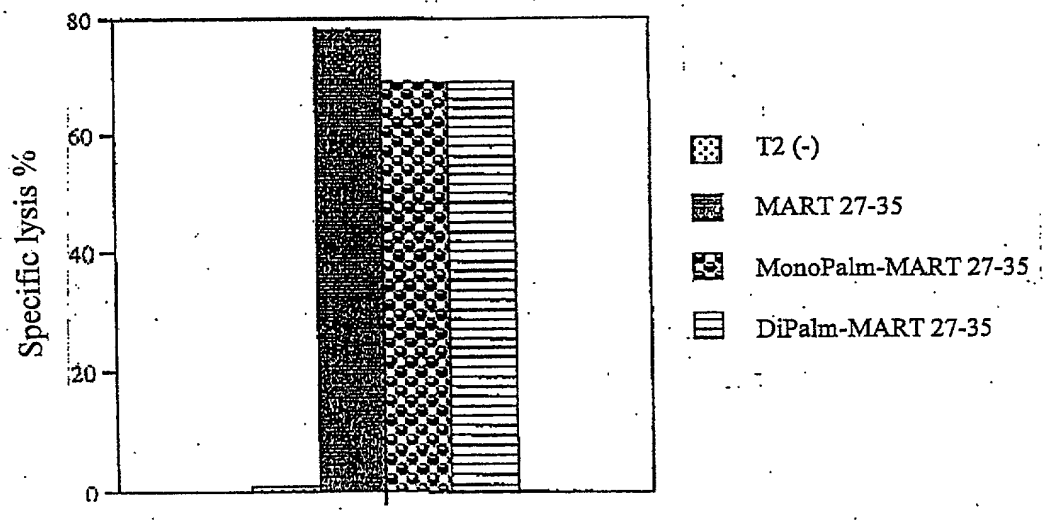


FIGURE 2B

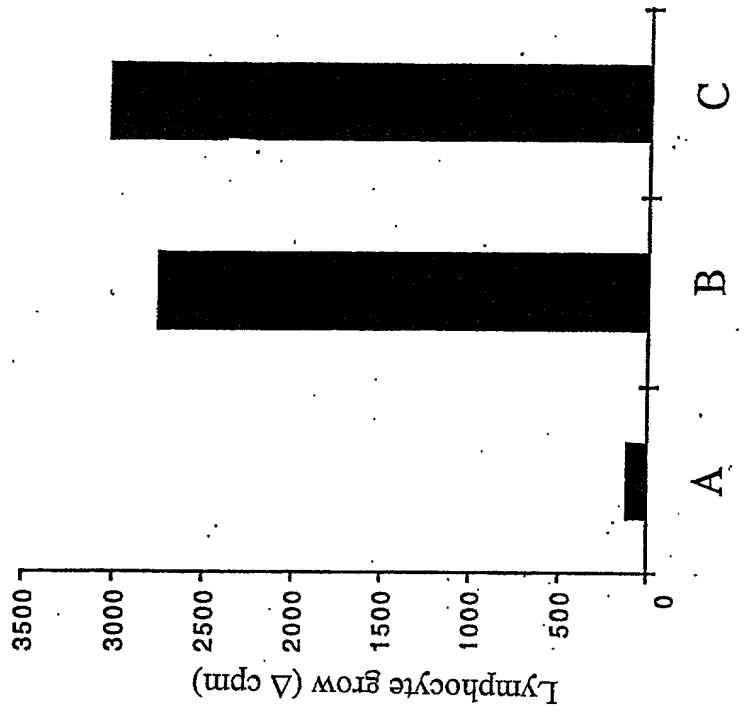


FIGURE 3B

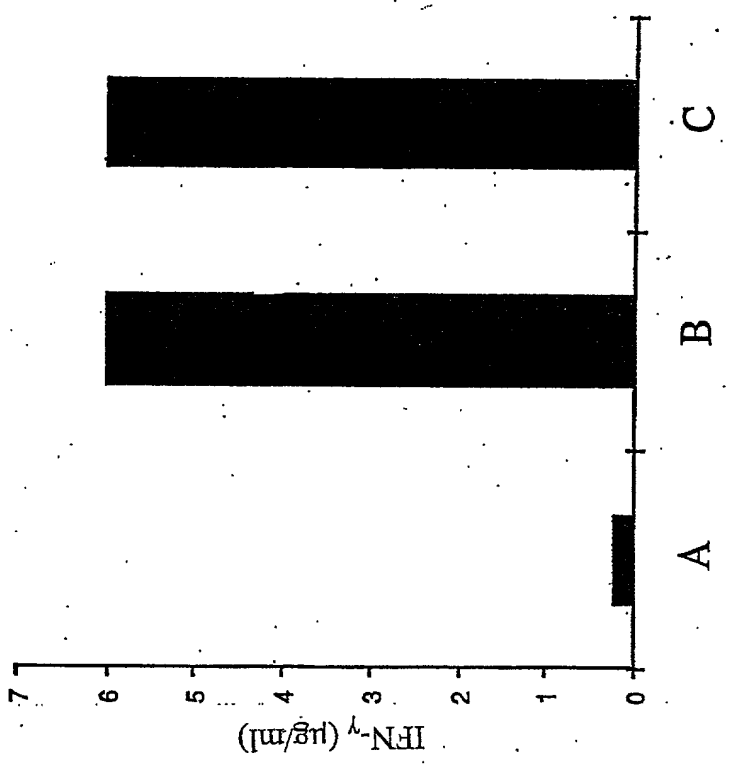


FIGURE 3A

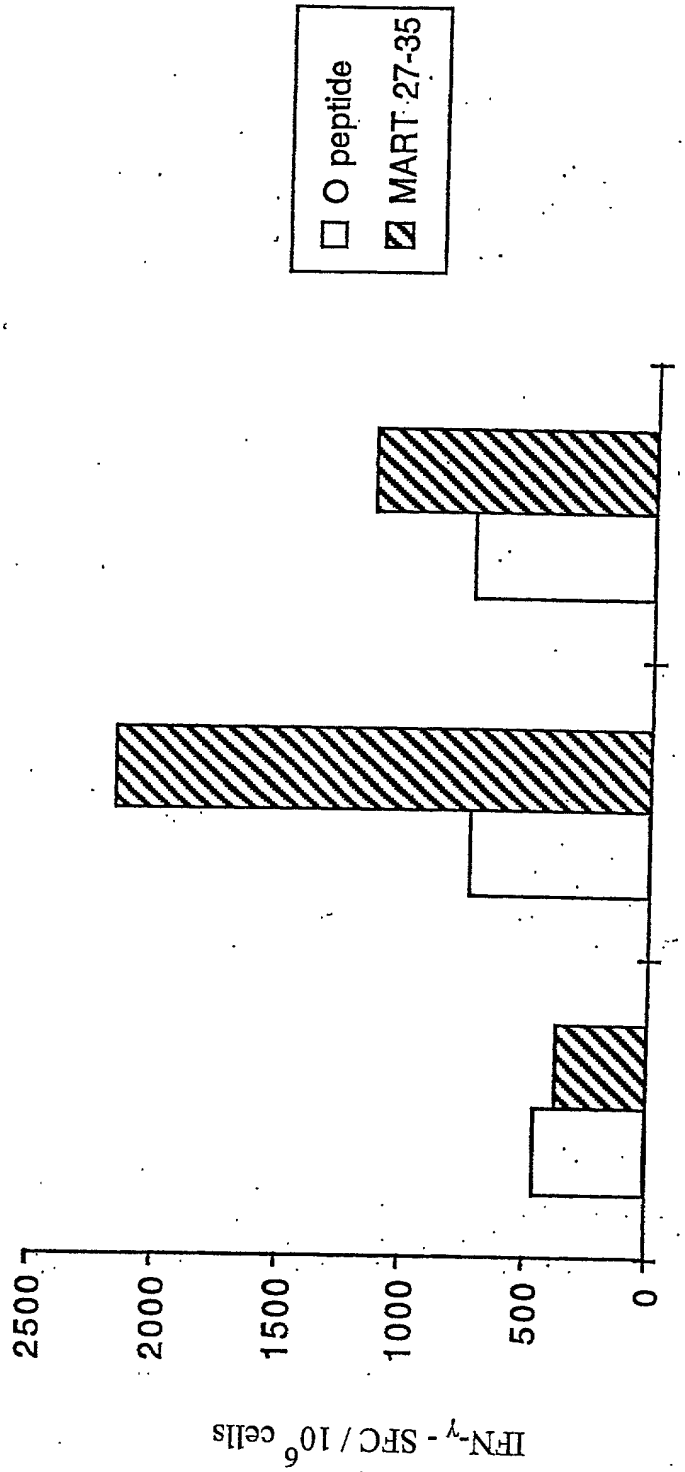

O peptide
MART 27-35
Dp-MART
Mp-MART
MART 27-35 + TT
Immunization
IFN-γ - SFC / 10^6 cells
2500
2000
1500
1000
500
0

FIGURE 4

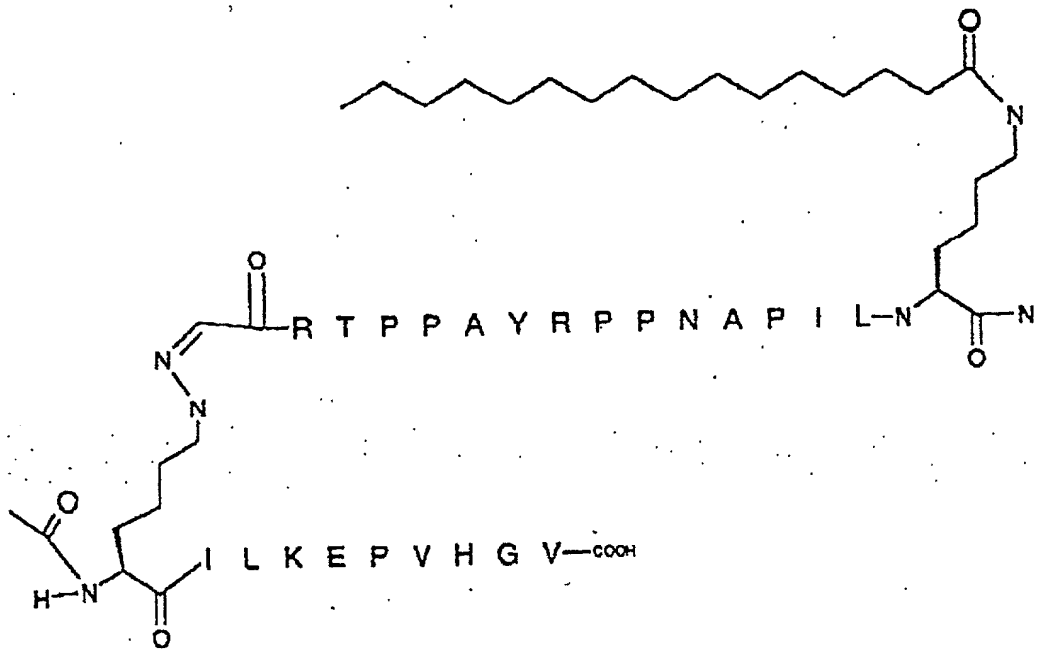
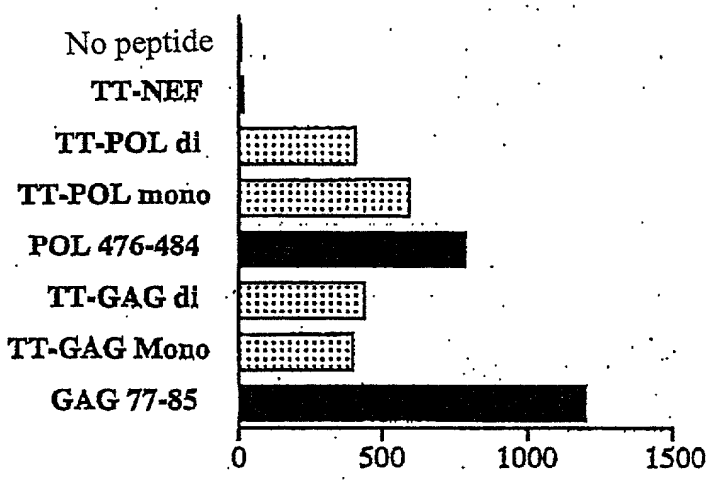
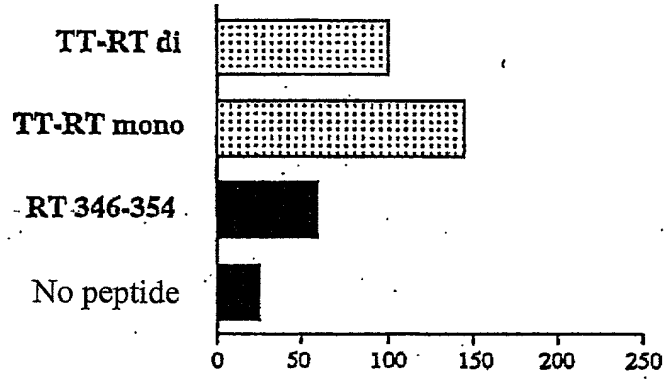


FIGURE 5

HLA-A2



Z 77



Z 108

Spot number for 10^6 cells

FIGURE 6A

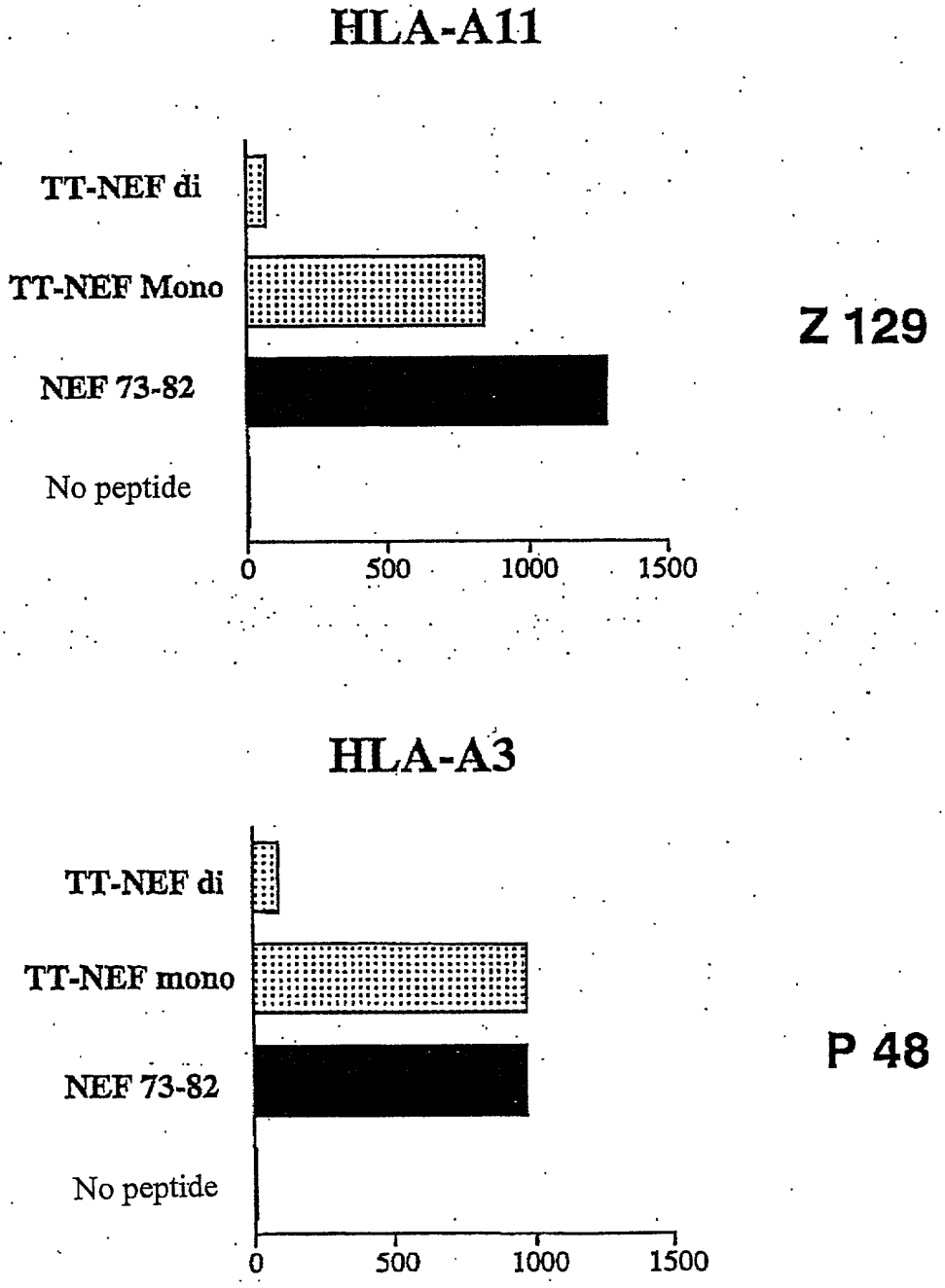
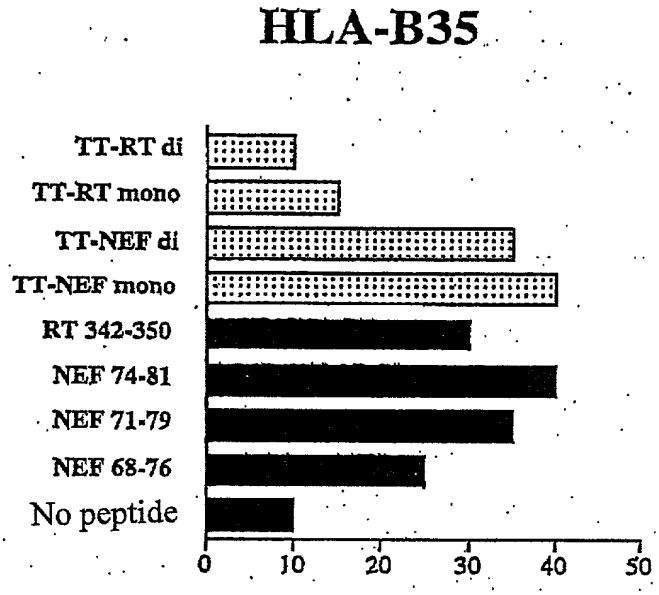

HLA-A11
TT-NEF di
TT-NEF Mono
NEF 73-82
No peptide
0
500
1000
1500
Z 129
HLA-A3
TT-NEF di
TT-NEF mono
NEF 73-82
No peptide
0
500
1000
1500
P 48
Spot number for 10^6 cells

FIGURE 6B


HLA-B35
TT-RT di
TT-RT mono
TT-NEF di
TT-NEF mono
RT 342-350
NEF 74-81
NEF 71-79
NEF 68-76
No peptide
0
10
20
30
40
50
P 3

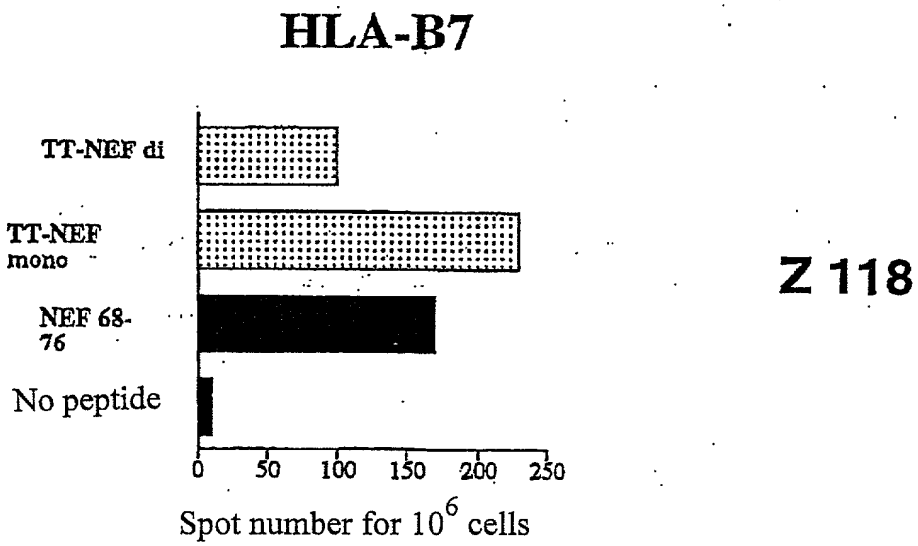

HLA-B7
TT-NEF di
TT-NEF mono
NEF 68-76
No peptide
0
50
100
150
200
250
Spot number for 10^6 cells
Z 118

FIGURE 6C

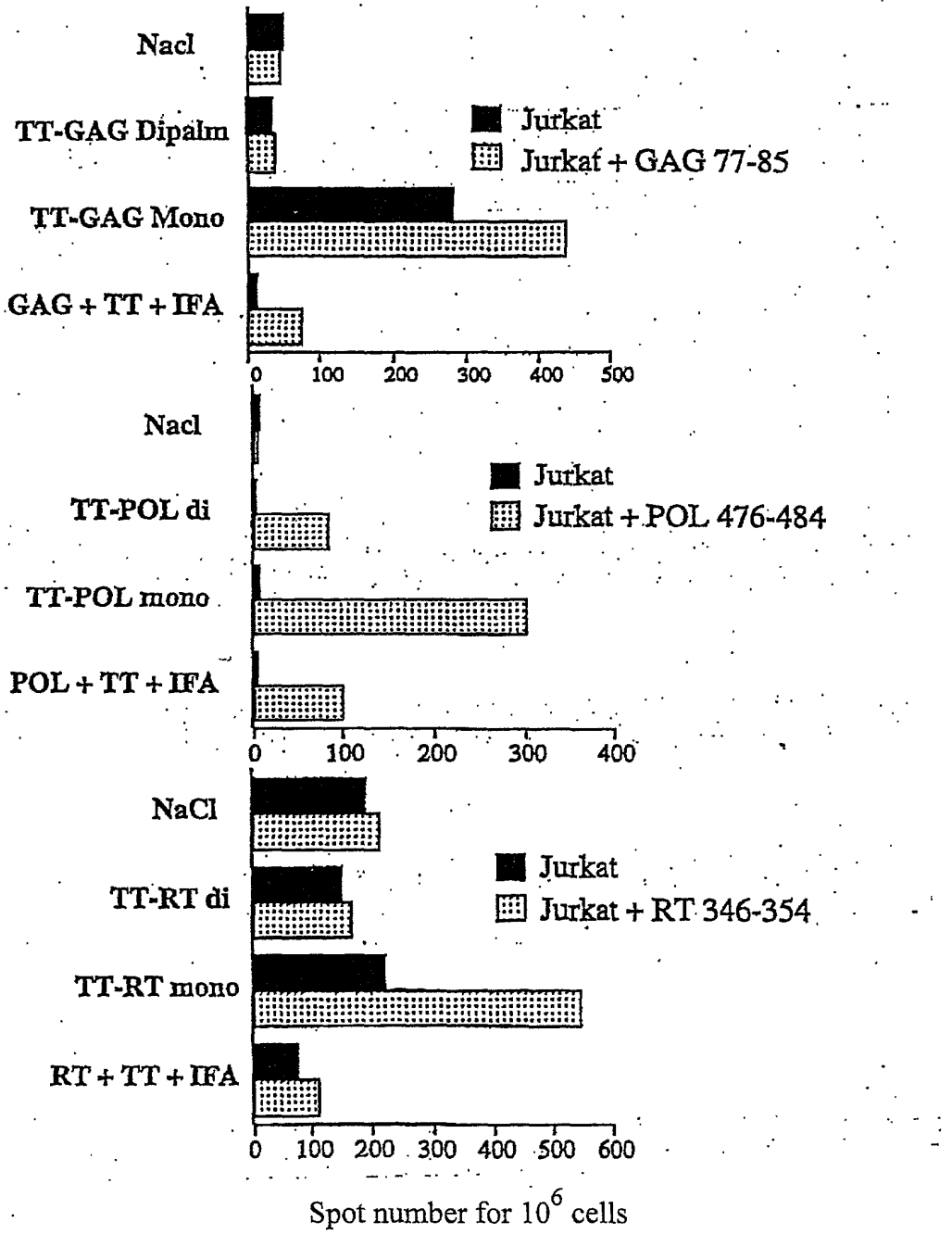

Nacl
TT-GAG Dipalm
TT-GAG Mono
GAG + TT + IFA
Jurkat
Jurkat + GAG 77-85
0 100 200 300 400 500
Nacl
TT-POL di
TT-POL mono
POL + TT + IFA
Jurkat
Jurkat + POL 476-484
0 100 200 300 400
NaCl
TT-RT di
TT-RT mono
RT + TT + IFA
Jurkat
Jurkat + RT 346-354
0 100 200 300 400 500 600
Spot number for 10^6 cells

FIGURE 7A

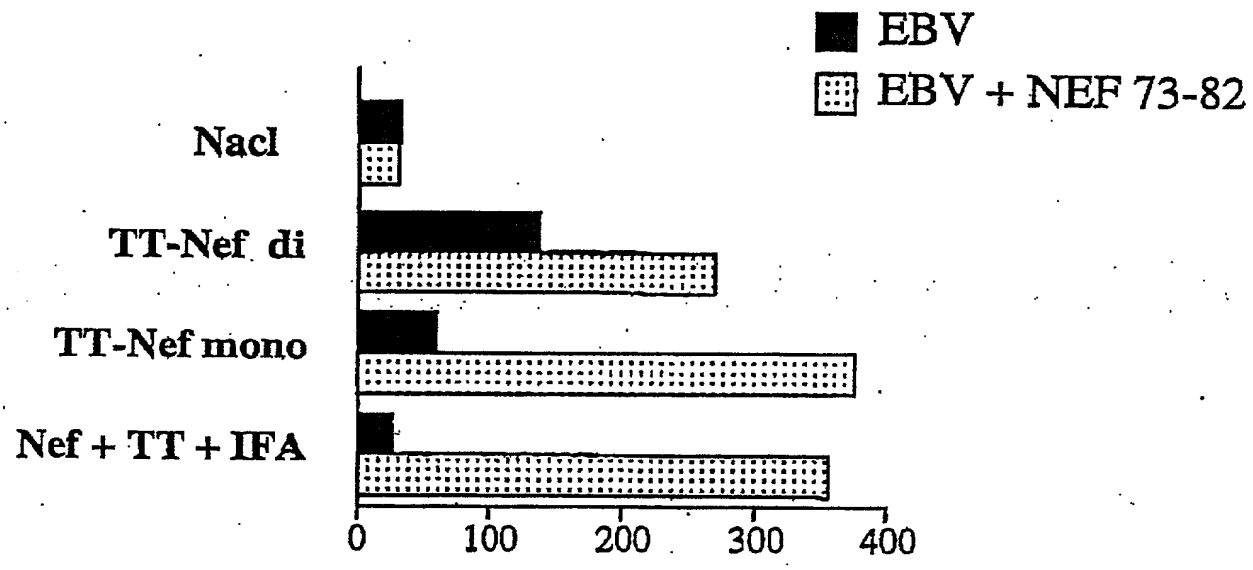

EBV
EBV + NEF 73-82
Nacl
TT-Nef di
TT-Nef mono
Nef + TT + IFA
0
100
200
300
400
Spot number for 10^6 cells

FIGURE 7B

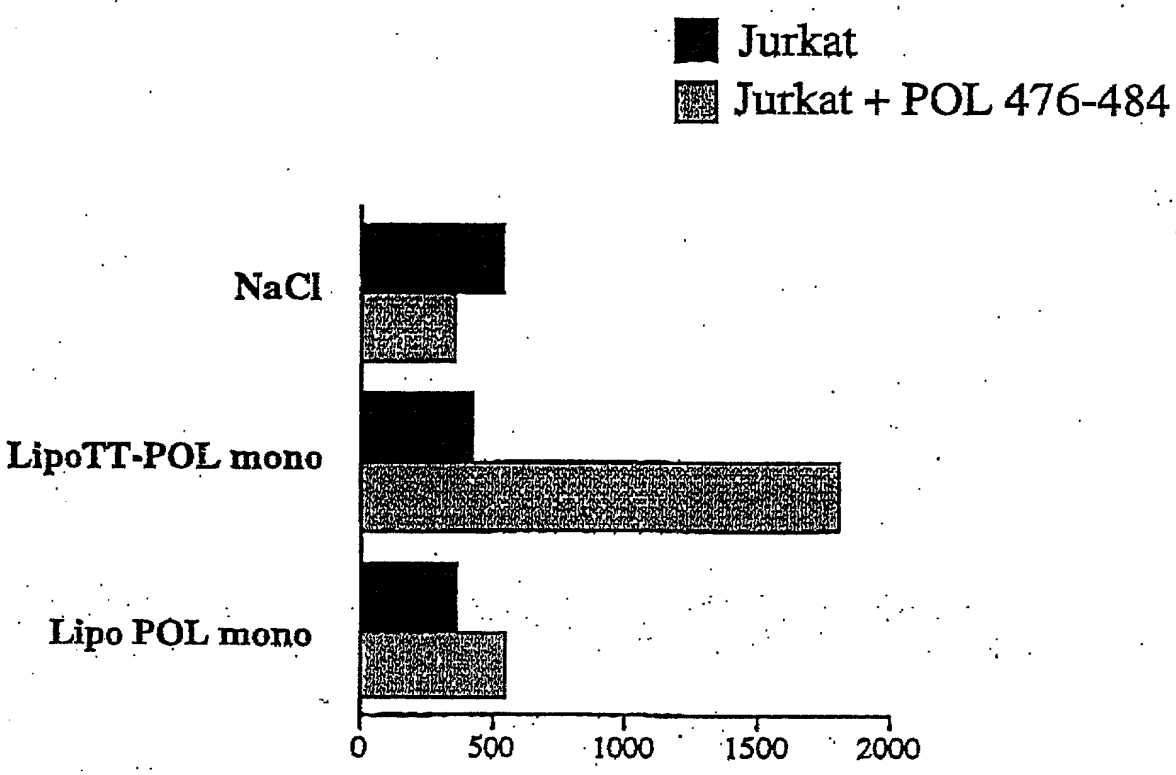

Jurkat
Jurkat + POL 476-484
NaCl
LipoTT-POL mono
Lipo POL mono
0
500
1000
1500
2000
Spot number for 10^6 cells

FIGURE 8A

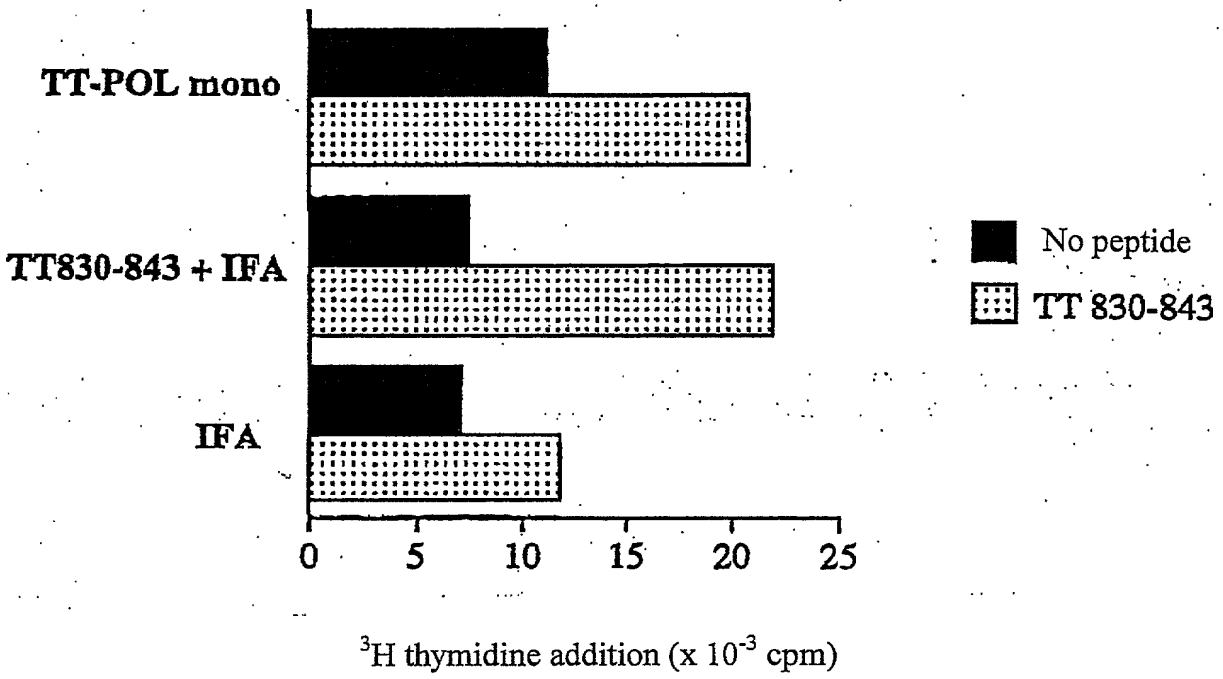

TT-POL mono
TT830-843 + IFA
IFA
0
5
10
15
20
25
No peptide
TT 830-843
^{3}H thymidine addition (x 10^{-3} cpm)

FIGURE 8B

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing

Attorney Docket Number	102.174
First Named Inventor	LE GAL et al
COMPLETE IF KNOWN	
Application Number	PCT/FR99/00792
Filing Date	April 6, 1999
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LIPOPEPTIDES INDUCING T LYMPHOCYTIC CYTOTOXICITY BEARING AT LEAST ONE AUXILIARY T EPITOPE, AND USES FOR VACCINATION

(Title of the invention)

the specification of which

[ ] is attached hereto
OR
[X] was filed on (MM/DD/YYYY) 04/06/99 as United States Application Number or PCT International

Application Number PCT/FR99/00792 and was amended on (MM/DD/YYYY) ______ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56

I hereby claim foreign priority benefits under Title 35, United States Code §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
98 04323	France	04/07/98	[ ]	[ ]	[X]
PCT/FR99/00792	PCT	04/06/99	[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority sheet attached hereto;

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

Burden Hour Statement: This form is estimated to take 0.4 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner of Patents and ...

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DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Name	Registration Number	Name	Registration Number
Bierman, Muserlian and Lucas	18,818		
Jordan B. Bierman	18,629		
Charles A. Muserlian	19,683		
Donald C. Lucas	31,275		

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

Direct all correspondence to:

Name	Charles A. Muserlian
Address	Bierman, Muserlian and Lucas
Address	600 Third Avenue
City	New York; State: NY; ZIP: 10016
Country	U.S.A.; Telephone: (212) 661-8000; Fax: (212) 661-8002

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name	Frederique Ahne
Middle Initial	
Family Name	LE GAL
Suffix e.g. Jr.	
Inventor's Signature	X [signature]
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Citizenship	France
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State	
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Country	France

☒ Additional inventors are being named on supplemental sheet(s) attached hereto

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DECLARATION

ADDITIONAL INVENTOR(S) Supplemental Sheet

2-00

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name	Middle Initial	Family Name	Suffix e.g. Jr.
Jean Gerard		GUILLET	

Inventor's Signature: X [signature] Date: X 11/30/2000

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Post Office Address:

City: Vanves State: Zip: F-92170 Country: France

3-00

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Given Name	Middle Initial	Family Name	Suffix e.g. Jr.
Hasine		GAHERY-SEGARD	

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City: Paris State: Zip: F-75014 Country: France

4-00

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name	Middle Initial	Family Name	Suffix e.g. Jr.
Helene		CRAS-MASSE	

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City: Merignies State: Zip: F-59710 Country: France

5-00

Name of Additional Joint Inventor, if any: ☐ A petition has been filed for this unsigned inventor

Given Name	Middle Initial	Family Name	Suffix e.g. Jr.
Oleg		MELNYK	

Inventor's Signature: X Oleg Melnyk Date: X 12/04/2000

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Post Office Address:

City: MONS-en-BAROEUL State: Zip: F-59370 Country: France

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DECLARATION

ADDITIONAL INVENTOR(S) Supplemental Sheet

6-00

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor				
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Inventor's Signature: [signature]	Date: 28-3-0				
Residence: City: VITRY-en-ARTOIS	State:	Country: France FRX	Citizenship: France		
Post Office Address: 1 rue du Marlin, 62490 VITRY-en-ARTOIS, France					
Post Office Address:					
City: VITRY-en-ARTOIS	State:	Zip: F-62490	Country: France		

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor		
Given Name:	Middle Initial:	Family Name:	Suffix e.g. Jr.
Inventor's Signature:	Date:		
Residence: City:	State:	Country:	Citizenship:
Post Office Address:			
Post Office Address:			
City:	State:	Zip:	Country:

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor		
Given Name:	Middle Initial:	Family Name:	Suffix e.g. Jr.
Inventor's Signature:	Date:		
Residence: City:	State:	Country:	Citizenship:
Post Office Address:			
Post Office Address:			
City:	State:	Zip:	Country:

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor		
Given Name:	Middle Initial:	Family Name:	Suffix e.g. Jr.
Inventor's Signature:	Date:		
Residence: City:	State:	Country:	Citizenship:
Post Office Address:			
Post Office Address:			
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